A Tissue Engineering product development pathway

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Thesis

A Tissue Engineering product development pathway

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Dedicated to Kasia

Additional dedications

This work is dedicated to my family, who always supported my choices, even when painful.

I thank all the Industrial Bioengineering Group, because they were my friends, not only my colleagues, without them it would not have been possible to survive my moments of despair in carrying out this work.

I will never forget the “pubetto” group, Marco Deriu, Giacomo Di Benedetto and Umberto Morbiducci for so many scientific meetings having alcoholic outcome at Charlie Bird, and Diana Massai for showing me the right way when I was wrong.

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“S'io avessi, lettor, più lungo spazio
da scrivere, i' pur cantere' in parte
lo dolce ber che mai non m'avrìa sazio;

ma perché piene son tutte le carte
ordite a questa cantica seconda,
non mi lascia più ir lo fren de l'arte.

Io ritornai da la santissima onda
rifatto sì come piante novelle
rinnovellate di novella fronda,
puro e disposto a salire a le stelle.”

Dante Alighieri, “Divina Commedia – Purgatorio – Canto XXXIII”

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Nomenclature

A = cross sectional area

$d_{p\text{-}compress}$ = mean pore size of the compressed scaffold

ΔP = differential pressure

ε = porosity

ε_e = effective porosity

φ = sphericity

H = hydraulic conductivity

i = interconnectivity

k = permeability

k_2 = non-darcian permeability

$\mathrm{K_{ij}}$ = permeability tensor

L = scaffold thickness

l = individual strut length

LAC = lacunarity

$\dot{\mathrm{M}}_{\mathrm{B1}}$ = mass flow rate without scaffold

$\dot{\mathrm{M}}_{\mathrm{B2}}$ = mass flow rate with scaffold

μ = viscosity

$\tilde{\mu}$ = effective viscosity

n_p = number of pores per unit area

η_{app} = applied compressive strain

P = pressure

Q = flow rate

R = hydraulic radius

Re_i = interstitial Reynolds number

r_f = fibre radius

r_g = grain radius

r_p = pore radius

R_w = radius of the water outlet

ρ = density

$\frac{\rho^*}{\rho_s}$ = scaffold relative density

S = fluid mobility

s = specific surface area per bulk volume

s_p = pore size

t = time

T = tortuosity

U = mean velocity - volumetric flow rate per unit of cross sectional area

w = radius of the scaffold sample

Introduction

Tissue science and engineering is the use of physical, chemical, biological, and engineering processes to control and direct the aggregate behaviour of cells. An overlapping field, regenerative medicine, encompasses some of the knowledge and practice of tissue science and engineering but also includes self-healing through endogenous recruitment or exogenous delivery of appropriate cells, biomolecules, and supporting structures.

The goal of tissue engineering is to repair or replace the damaged organ or tissues by delivering functional cells, supporting scaffolds, growth promoting, and signal molecules or DNA encoding these molecules to areas. The field has already made headway in the synthesis of structural tissues such as skin, cartilage, bone, and bladder.

The classic tissue engineering strategy is to isolate specific cells through a biopsy from a patient, to grow them on a three-dimensional (3D) biomimetic scaffold under precisely controlled culture conditions, to deliver the construct to the desired site in the patient's body, and to direct new tissue formation into the scaffold that can be degraded over time (Fig. 1) (1).

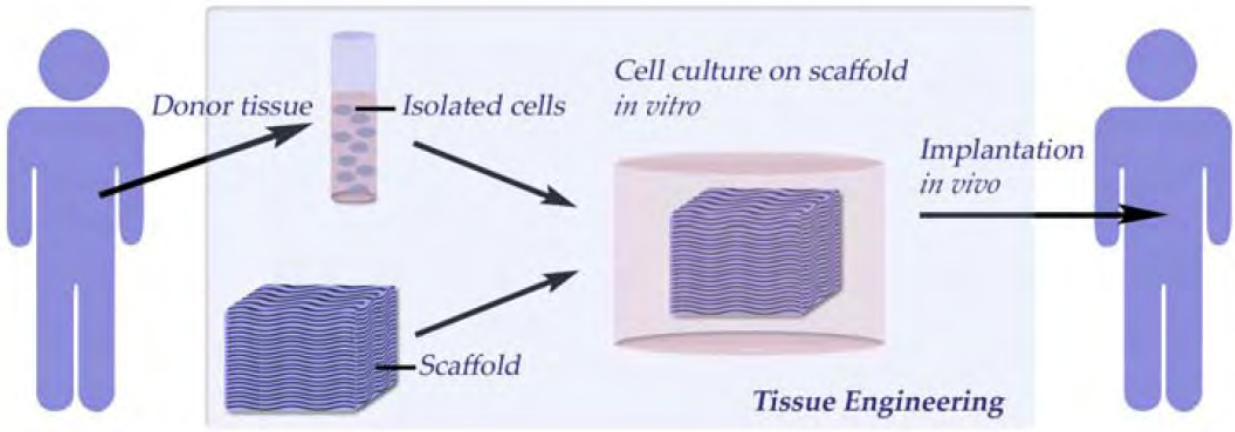


Fig. 1: Schematic illustration of tissue engineering principle (1).

In order to achieve successful regeneration of damaged organs or tissues based on the tissue engineering concept, several critical elements should be considered, including the scaffold that serves as a mechanical and biological support for cell growth and differentiation, progenitor cells that can be differentiated into specific cell types, and the inductive growth factors that can modulate cellular activities. Strategies of tissue engineering can be classified as in vitro and in vivo approaches.

One of the greatest promises of tissue engineering is the replacement of the function of whole defective organs. Up until now patient usually have been dependent on organs of living or dead donors. Although organ transplantation often is a life-saving, effective, and inevitable form of therapy, it still implies many practical and ethical problems such as lack of organs and therefore questions of justice and allocation, of pressure on possible donors, of altruism and duty, organ sale, xeno-transplantation and so on. Furthermore, there are problems of compatibility, graft failure, graft rejection, side effects of the immunosuppressive therapy, mental and economic costs, and related difficulties. Also the near connection to the controversial brain death criterion, the implied mechanical understanding of the human body and social implications often provoke uneasiness.

Tissue engineering could evade many of these difficulties. If enough organs could be bred without the need of donor organs, the considerations about ethically acceptable ways of enhancing the willingness to donation would be superfluous.

Tissue engineering also offers the opportunity of designing specific human cell-lines for testing the effects of new pharmaceuticals and therefore could replace (at least in parts) the urge of animal research with their sometimes questionable evidence concerning human beings. Systems are being developed in the field of reparative medicine to generate cell and tissue-based constructs. These systems that attempts to simulate a physiological environment for the creating of tissues in vitro are known as bioreactors (2).

The high degree of reproducibility, control and automation introduced by bioreactors for specific experimental bioprocesses has been key for their transfer to large-scale applications. Ex vivo engineering of cell-based grafts to restore damaged or diseased tissues has the potential to revolutionize current therapeutic strategies for severe physical disabilities and to improve the quality of life. One of the major challenges in reaching this ambitious goal is to translate research-scale production models into clinically applicable manufacturing designs that are reproducible, clinically effective and economically acceptable, while complying with Good Manufacturing Practice (GMP) requirement. Similar to pharmaceuticals, tissue-engineered products have multimillion-dollar expenditures associated with winning regulatory approval, but, unlike many pharmaceuticals, they have not been able to recoup the regulatory costs and enjoy the same high profit margins. Starting with a patient's tissue biopsy, a bioreactor system could isolate, expand, seed specific cell types on a scaffold and culture the resulting construct until a suitably developed graft is generated, thereby performing the different processing phases within a single closed and automated system (Fig. 2). Bioreactor would enable competent hospitals and clinics to carry out autologous tissue engineering for their own patients, eliminating logistical issues of transferring specimens between locations. This would also eliminate the need for large and expensive GMP tissue engineering facilities and minimize operator handling, with the final result of reducing the cost of tissue engineered products for the health system and for the community (3).

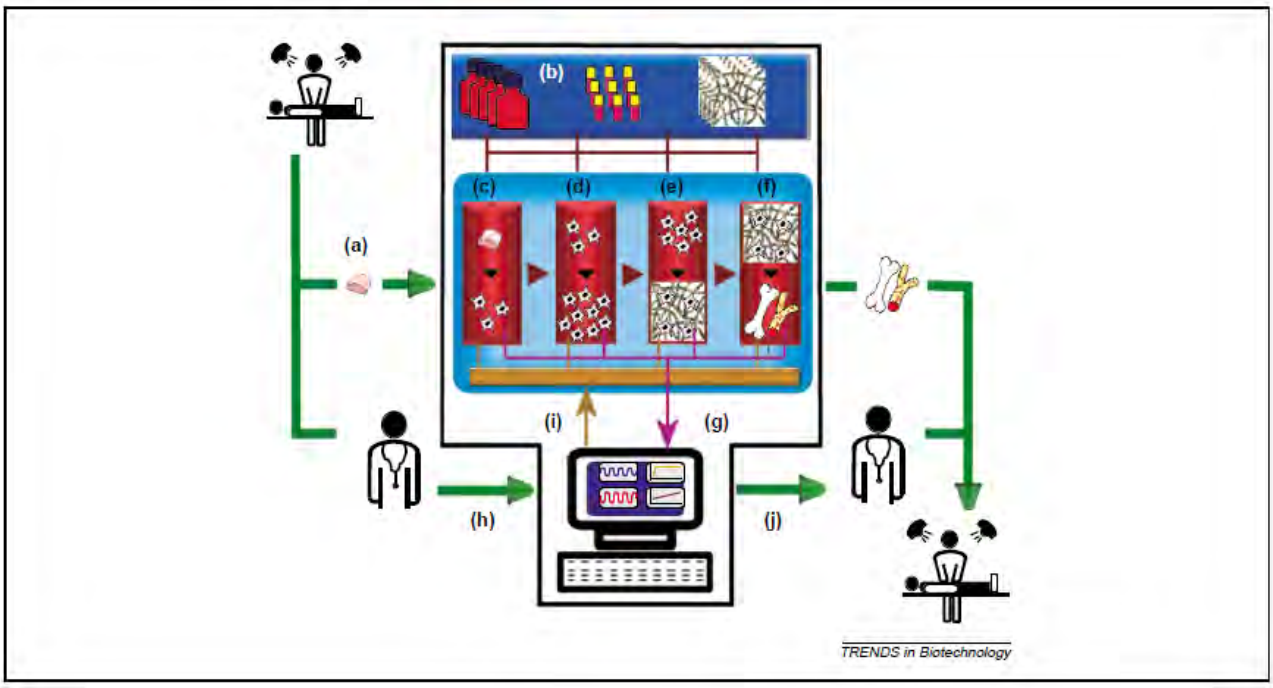


Fig. 2: Vision for a closed-system bioreactor for the automated production of tissue engineered grafts. (a) The surgeon would take a biopsy from the patient and introduce it into the bioreactor located on-site at the hospital. (b) All reagents (e.g. culture

medium, medium supplements, and scaffolds) would be stored in compartments under appropriate conditions (i.e. temperature, humidity). The bioreactor system could then (c) automatically isolate the cells,(d) expand the cells,(e)seed the cells onto a scaffold , and (f) culture the construct until a suitably developed graft is produced. (g) Environmental culture parameters and tissue development would be monitored and inputs fed into a microprocessor unit for analysis. In conjunction with data derived from clinical records of the patient (h), the inputs would be used to control culture parameters at pre-defined optimum levels automatically (i) and provide the surgical team with data on the development of the tissue, enabling timely planning of the implantation (j) (3).

The big opportunity supplied by tissue engineering products is hampered by the fact that an effective and economically safe development pathway has not been assessed yet.

First of all, big companies are only slightly involved in the development of this kind of product that are usually prerogative of small/medium enterprises. This falls in a very wide and patchy diffusion of competitors, but, as other side of the coin, a less aggressive competition and less of barriers of entry related to big companies' competition. So, tissue engineering product development is likely to be more easily feasible starting from a small/medium enterprise.

Basic principle of an effective and affordable product development is to identify the resources to be used, both in terms of funds and in terms of work. So, fund raising and "workforce" raising are critical aspects.

As any product, in the field of healthcare, having a large impact on population's longevity and quality of life, mainly if the pathologies in which that product is utilized are widely diffused and highly mortal, but having long time to be marketed and to supply reimbursement, and strongly limited by regulation as well, the interest tissue engineering products can instil on public institution is far higher (and so, far more exploitable) than private companies' one. For instance, the European Community has favoured and financed public and industrial research projects in this field for the last ten years. As a consequence, fund raising strategy must absolutely gravitate around public funding.

Moreover, it is important to consider that the economic strength of any company, a fortiori of a small/medium enterprise, is based on reimbursement its products give, so the most part of a company's R&D effort should be focused on the development of immediately marketable products, and tissue engineering products cannot certainly be framed in this definition. This leads to the need of outsourcing the development of this kind of product. A smart strategy of "workforce" raising will be to let public research centres and university achieve this task instead of internal R&D departments. This strategy will have the further advantage of increasing the probability of receiving public funding because the involvement of public entities in private research and development project usually raises the score during the evaluation of the project by public commissions.

A strong industrial protection can be reached by isolating the development of these cutting-edge products from the core business of a company using industrial start-ups, so limiting the probability of failure.

Finally, keeping in mind that rules are the worst enemy of product development, among several products developable, it is advisable to follow the pathway that are less hampered by regulatory claims. So, first of all, it is compulsory to avoid cell-seeded products. In fact, these products would fall in the category of "advanced therapy medicinal products", making certification long-lasting and hugely expensive, affordable just either by big companies, or by start-ups of big companies and spin-offs of complex aggregates of research centres involved in advanced cell research.

So, in this pathway, two kind of products are kept under consideration, namely (i) unseeded scaffolds for tissue engineering (certification class IIa, IIb and III, and (ii) bioreactors for tissue engineering (certification class I and IIa).

Objective of this thesis

This work aims at developing products in a cutting-edge field, namely tissue engineering, using small/medium enterprises as a launch pad.

First, a selection of products that can be affordably developed must be carried out, paying attention to regulatory claims too, being the latter a prevailing reason of failure. Avoiding cell-seeded products is a key point, because they fall in the category of “advanced therapy medicinal products”, making certification long-lasting and hugely expensive, so unbearable by small/medium enterprises.

A preliminary study of tissue engineering is needed, in order to focus this subject, followed by the development of a few prototypes.

Then, a specific product development model must be elaborated, identifying the resources to be used, both in terms of funds and in terms of work, provided that fund raising and “workforce” raising are critical aspects in order to reach this objective in the quickest and most cost-effective way. Particularly, the model that will be developed aims at (i) setting a fund raising strategy based on public funding and (ii) setting a “workforce” raising strategy based on strategic outsourcing of R&D activities to Universities and Research Centres.

Finally, after showing the correct working of prototypes, the effectiveness of the product development model must be assessed.

1. Basic research on scaffolds

One of the principle methods behind tissue engineering involves growing the relevant cell(s) in vitro into the required three-dimensional (3D) organ or tissue. But cells lack the ability to grow in favoured 3D orientations and thus define the anatomical shape of the tissue. Instead, they randomly migrate to form a two-dimensional (2D) layer of cells. However, 3D tissues are required and this is achieved by seeding the cells onto porous matrices, known as scaffolds, to which the cells attach and colonise (4). The scaffold therefore is a very important component for tissue engineering (5).

Several requirements have been identified as crucial for the production of tissue engineering scaffolds (6):

- the scaffold should possess interconnecting pores of appropriate scale to favour tissue integration and vascularisation,
- be made from material with controlled biodegradability or bioresorbability so that tissue will eventually replace the scaffold,
- have appropriate surface chemistry to favour cellular attachment, differentiation and proliferation,
- possess adequate mechanical properties to match the intended site of implantation and handling,
- should not induce any adverse response and,
- be easily fabricated into a variety of shapes and sizes.

Bearing these requirements in mind, several materials have been adopted or synthesised and fabricated into scaffolds

In order for biological cells to maintain tissue-specific functions, a porous substrate, called scaffold, must be used to provide a simulated environment for dissociated cells to grow and reform.

Natural polymers, such as proteins and polysaccharides, have also been used for tissue engineering applications (7).

Collagen is a fibrous protein and a major natural extracellular matrix component. It has been used for various tissue regeneration applications, especially for soft tissue repair (8), (9). On one hand, collagen as a natural extracellular component has useful biological properties desirable for tissue engineering applications. Therefore, collagen-glycosaminoglycan (GAG) copolymers are fabricated into scaffolds for tissue engineering (10),(11). Denatured collagen (gelatin) is also processed into porous materials for tissue repair(12). On the other hand, there are concerns over collagen because of the potential pathogen transmission, immune reactions, poor handling and mechanical properties, and less controlled biodegradability (7).

Another category of well-known natural fibrous proteins is silk. Silkworm silk has been used in textile production for centuries, and has been used as nondegradable sutures for decades because of its excellent tensile mechanical properties (13). This natural macromolecular material has recently been introduced into the field of tissue engineering (14). Although silk is often characterized as a nondegradable material, it can degrade in vivo via enzymatic mechanisms. However, the degradation rate is very slow. There is also some concern over cytotoxicity (15). There is research into the chemical modification of silk materials to enhance biocompatibility. There are also other types of silks, such as spider silks and even genetically engineered silks(16), which may be considered in certain applications (7).

Polysaccharides are another class of natural polymers. For example, alginate(17), chitosan(18), and hyaluronate(19) have been used as porous solid-state tissue engineering scaffolds (7).

In addition to the use of relatively pure natural macromolecules extracted from an animal or plant tissue source, processed extracellular matrix (decellularized) materials with multiple natural macromolecules are also used as scaffolds for tissue engineering or repair applications (7). One such example is small intestinal submucosa (SIS), which contains type I collagen, GAGs, and some growth factors. SIS has been used in the reconstruction of several tissue types(20),(21). Similarly, other decellularized tissues, such as urinary bladder submucosa, porcine heart valves, and human dermis have been used for tissue engineering or repair(22)-(23). Again there are concerns over pathogen transmission and immune rejection (24).

1.1. Collagen, a versatile material for scaffolds

1.1.1. Molecular and Fibrillar Structure

The *tropocollagen* or "collagen molecule" (Fig. 3) is a subunit of larger collagen aggregates such as fibrils. It is approximately 300 nm long and 1.5 nm in diameter, made up of three polypeptide strands (called alpha peptides), each possessing the conformation of a left-handed helix (its name is not to be confused with the commonly occurring alpha helix, a right-handed structure). These three left-handed helices are twisted together into a right-handed coiled coil, a triple helix or "super helix", a cooperative quaternary structure stabilized by numerous hydrogen bonds. With type I collagen and possibly all fibrillar collagens if not all collagens, each triple-helix associates into a right-handed super-super-coil that is referred to as the collagen microfibril. Each microfibril is interdigitated with its neighboring microfibrils to a degree that might suggest that they are individually unstable although within collagen fibrils they are so well ordered as to be crystalline(25).

A distinctive feature of collagen is the regular arrangement of amino acids in each of the three chains of these collagen subunits. The sequence often follows the pattern Glycine-Proline-Y or Glycine-X-Hydroxyproline, where X and Y may be any of various other amino acid residues. Proline or hydroxyproline constitute about 1/6 of the total sequence. With Glycine accounting for the 1/3 of the sequence, this means that approximately half of the collagen sequence is not proline or hydroxyproline, a fact often missed due to the distraction of the unusual GXY character of collagen alpha-peptides. This kind of regular repetition and high glycine content is found in only a few other fibrous proteins, such as silk fibroin. 75-80% of silk is (approximately) -Glycine-Alanine-Glycine-Alanine- with 10% serine—and elastine is rich in glycine, proline, and alanine, whose side group is a small, inert methyl group. Such high glycine and regular repetitions are never found in globular proteins save for very short sections of their sequence. Chemically-reactive side groups are not needed in structural proteins as they are in enzymes and transport proteins, however collagen is not quite just a structural protein. Due to its key role in the determination of cell phenotype, cell adhesion, tissue regulation and infrastructure, many sections of its non-proline rich regions have cell or matrix association/regulation roles. The relatively high content of proline and hydroxyproline rings, with their geometrically constrained carboxyl and (secondary) amino groups, along with the rich abundance of glycine, accounts for the tendency of the individual polypeptide strands to form left-handed helices spontaneously, without any intrachain hydrogen bonding(25).

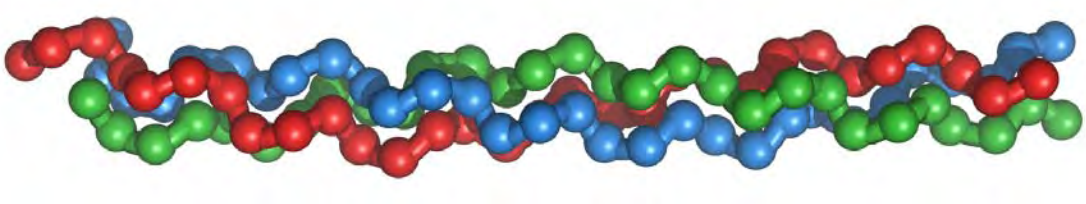


Fig. 3: Tropocollagen triple helix.

The tropocollagen subunits spontaneously self-assemble, with regularly staggered ends, into even larger arrays in the extracellular spaces of tissues(25),(26). In the fibrillar collagens, the molecules are staggered from each other by about 67nm (a unit that is referred to as 'D' and changes depending upon the hydration state of the aggregate). Each D-period contains 4 and a fraction collagen molecules. This is because 300 nm divided by 67 nm does not give an integer (the length of the collagen molecule divided by the stagger distance D). Therefore in each D-period repeat of the microfibril, there is a part containing 5 molecules in cross-section – called the "overlap" and a part containing only 4 molecules(27). The triple-helices are also arranged in a hexagonal or quasi-hexagonal array in cross-section, in both the gap and overlap regions(27),(28).

There is some covalent crosslinking within the triple helices, and a variable amount of covalent crosslinking between tropocollagen helices forming well organized aggregates (such as fibrils)(29). Larger fibrillar bundles are formed with the aid of several different classes of proteins (including different collagen types), glycoproteins and proteoglycans to form the different types of mature tissues from alternate combinations of the same key players(25). Collagen's insolubility was a barrier to the study of monomeric collagen until it was found that tropocollagen from young animals can be extracted because it is not yet fully crosslinked. However, advances in microscopy techniques (Electron Microscopy - EM and Atomic Force Microscopy - AFM) and X-ray diffraction have enabled researchers to obtain increasingly detailed images of collagen structure *in situ*. These later advances are particularly important to better understanding the way in which collagen structure affects cell-cell and cell-matrix communication and how tissues are constructed in growth and repair, and changed in development and disease(30),(31).

Collagen fibrils are collagen molecules packed into an organized overlapping bundle. Collagen fibers are bundles of fibrils.

Collagen fibrils/aggregates are arranged in different combinations and concentrations in various tissues to provide varying tissue properties. In bone, entire collagen triple helices lie in a parallel, staggered array. 40 nm gaps between the ends of the tropocollagen subunits probably serve as nucleation sites for the deposition of long, hard, fine crystals of the mineral component, which is (approximately) hydroxyapatite, $Ca_{10}(PO_4)_6$ $(OH)_2$with some phosphate. It is in this way that certain kinds of cartilage turn into bone. Type I collagen gives bone its tensile strength.

1.1.2. Types of collagen and associated disorders

Collagen occurs in many places throughout the body. There are more than 28 types of collagen described in literature. Over 90% of the collagen in the body, however, are of type I, II, III, and IV.

- Collagen One - skin, tendon, vascular, ligature, organs, bone (main component of bone)
- Collagen Two - cartilage (main component of cartilage)
- Collagen Three - reticulate (main component of reticular fibers), commonly found alongside type I.
- Collagen Four - forms bases of cell basement membrane

Among the isotypes of collagen, type I is the principal structural and functional protein and is composed of two α1 chains and one α2 chain. The underlying a chains that form these natural polymers are arranged into a repeating motif that forms a coiled structure. The specific complement of a subunits present within the fibril defines the material properties of the polymer(32). In native tissues, type I collagen fibrils range from 50 to 500nm in diameter and are very uniform in size(32). The fibrillar structure of type I collagen has long been known to be important for cell attachment, proliferation, and differentiated function in tissue

culture (33), (34),(35). In native ECM, collagen exists in a three-dimensional network structure composed of multi-fibrils in the nanofiber scale (50–500 nm)(36), (37).

Collagen diseases commonly arise from genetic defects that affect the biosynthesis, assembly, postranslational modification, secretion, or other processes in the normal production of collagen.

Table 1: Collagen types and related disorders.

Type	Notes	Gene(s)	Disorders
I	This is the most abundant collagen of the human body. It is present in scar tissue, the end product when tissue heals by repair. It is found in tendons, skin, artery walls, the endomysium of myofibrils, fibrocartilage, and the organic part of bones and teeth.	COL1A1, COL1A2	osteogenesis imperfecta, Ehlers-Danlos Syndrome
II	Hyaline cartilage, makes up 50% of all cartilage protein. Vitreous humour of the eye.	COL2A1	Collagenopathy, types II and XI
III	This is the collagen of granulation tissue, and is produced quickly by young fibroblasts before the tougher type I collagen is synthesized. Reticular fiber. Also found in artery walls, skin, intestines and the uterus	COL3A1	Ehlers-Danlos Syndrome
IV	basal lamina; eye lens. Also serves as part of the filtration system in capillaries and the glomeruli of nephron in the kidney.	COL4A1, COL4A2, COL4A3, COL4A4, COL4A5, COL4A6	Alport syndrome
V	most interstitial tissue, assoc. with type I, associated with placenta	COL5A1, COL5A2, COL5A3	Ehlers-Danlos syndrome (Classical)
VI	most interstitial tissue, assoc. with type I	COL6A1, COL6A2, COL6A3	Ulrich myopathy and Bethlem myopathy
VII	forms anchoring fibrils in dermal epidermal junctions	COL7A1	epidermolysis bullosa dystrophica
VIII	some endothelial cells	COL8A1, COL8A2	-
IX	FACIT collagen, cartilage, assoc. with type II and XI fibrils	COL9A1,	- EDM2 and EDM3

		COL9A2, COL9A3	
X	Hypertrophic and mineralizing cartilage	COL10A1	Schmid metaphyseal dysplasia
XI	cartilage	COL11A1, COL11A2	Collagenopathy, types II and XI
XII	FACIT collagen, interacts with type I containing fibrils, decorin and glycosaminoglycans	COL12A1	-
XIII	transmembrane collagen, interacts with integrin a1b1, fibronectin and components of basement membranes like nidogen and perlecan.	COL13A1	-
XIV	FACIT collagen	COL14A1	-
XV	-	COL15A1	-
XVI	-	COL16A1	-
XVII	transmembrane collagen, also known as BP180, a 180 kDa protein	COL17A1	Bullous Pemphigoid and certain forms of junctional epidermolysis bullosa
XVIII	source of endostatin	COL18A1	-
XIX	FACIT collagen	COL19A1	-
XX	-	COL20A1	-
XXI	FACIT collagen	COL21A1	-
XXII	-	COL22A1	-
XXIII	MACIT collagen -	COL23A1	-
XXIV	-	COL24A1	-
XXV	-	COL25A1	-
XXVI	-	EMID2	-

XXVII	-	COL27A1	-
XXVIII	-	COL28A1	-
XXIX	epidermal collagen	COL29A1	Atopic Dermatitis(38)

In addition to the above mentioned disorders, excessive deposition of collagen occurs in Scleroderma.

1.1.3. Staining

In histology, collagen is brightly eosinophilic (pink) in standard H&E slides. The dye methyl violet may be used to stain the collagen in tissue samples.

The dye methyl blue can also be used to stain collagen and immunohistochemical stains are available if required.

The best stain for use in differentiating collagen from other fibers is Masson's trichrome stain.

1.1.4. Electrospun collagen nanofiber scaffolds

Size of Nanofibers and Porosity

Continuous collagen nanofibers were obtained at a concentration of 8% by weight in HFIP (Hexafluoroisopropanol, 1,1,1,3,3,3-hexafluoro-2-propanol), a concentration that appears to correspond to the onset of significant chain entanglements. The solution concentration of 8% by weight was chosen to fabricate a nonwoven matrix of randomly arranged collagen fibers with nanometer scale diameters. Fig. 4 shows a SEM micrograph of the as-spun (i.e., uncross-linked) collagen nanofibers under magnification of 5000x(37).

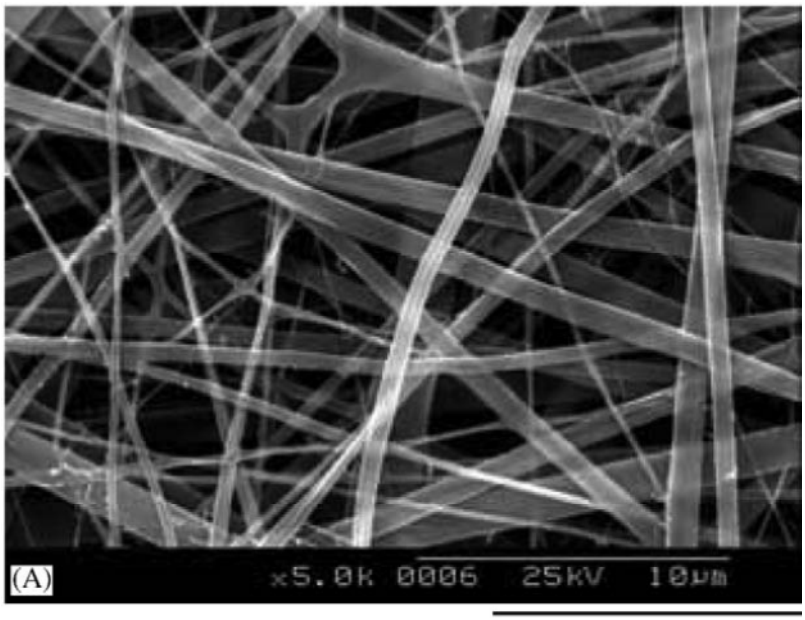


Fig. 4: SEM image of as-spun type I collagen nanofibers. Bar, 10 mm; 5000x magnification(37).

The as-spun collagen nanofibers were a somewhat less uniform matrix of fibers with a smooth surface. From the image analysis, it was found that the fibers had an average diameter of 460 nm, with a range of 100–1200 nm, as shown in Fig. 5(37).

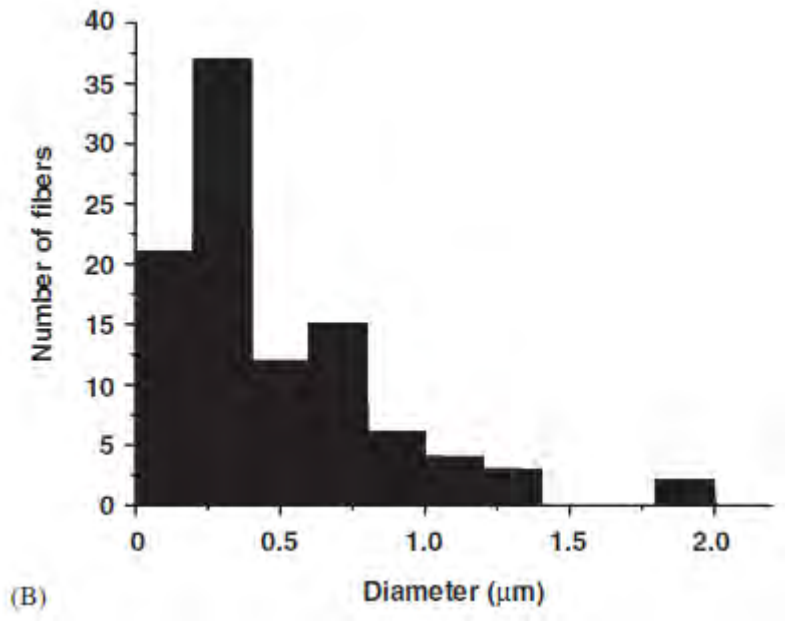


Fig. 5: Fiber diameter distribution of as-spun type I collagen nanofibers(37).

Pore parameters of as-spun and cross-linked collagen matrices determined by mercury porosimetry are summarized in Table 1(37).

Table 1: Mercury porosimetry analisys of as-spun and cross-linked collagen nanofibrous matrices (37).

Samples	TIV (ml/g)[1]	TPA (m^2/g)[2]	APD (μm)[3]			D (g/ml)[4]	P (%)[5]
			Volume (V)	Area (A)	4V/A		
As-spun	9.328	60.856	77.993	0.008	0.613	0.096	89.21
Cross-linked (12h)	1.566	39.452	135.989	0.007	0.159	0.452	70.83

The porosity of the as-spun collagen nanofibrous matrix was 89%, indicating that it was highly porous. The total pore volume was 9.328 ml/g, and the total pore area was 60.856 m^2/g. Whereas the bulk density of the matrix composed of collagen nanofibers increased from 0.096 to 0.452 g/ml during cross-linking for 12 h, the porosity and pore volume decreased from 89% to 71% and from 9.328 to 1.566 ml/g, respectively, indicating that the collagen matrix was distorted to form a denser structure by cross-linkage. However, we believe that the cross-linked collagen matrix had a higher dimensional stability in aqueous medium due to cross-linking for 12 h. From the deviation in average pore diameter in terms of volume (V), area (A), and 4V/A, it can be concluded that the pore size distribution was very broad. Fig. 6 shows the influence of cross-linking on the pore size distribution in the as-spun collagen matrix(37).

[1] TIV, total intrusion volume.
[2] TPA, total pore area.
[3] APD, average pore diameter; 4V/A, 4 volume/area.
[4] D, bulk density.
[5] P, porosity.

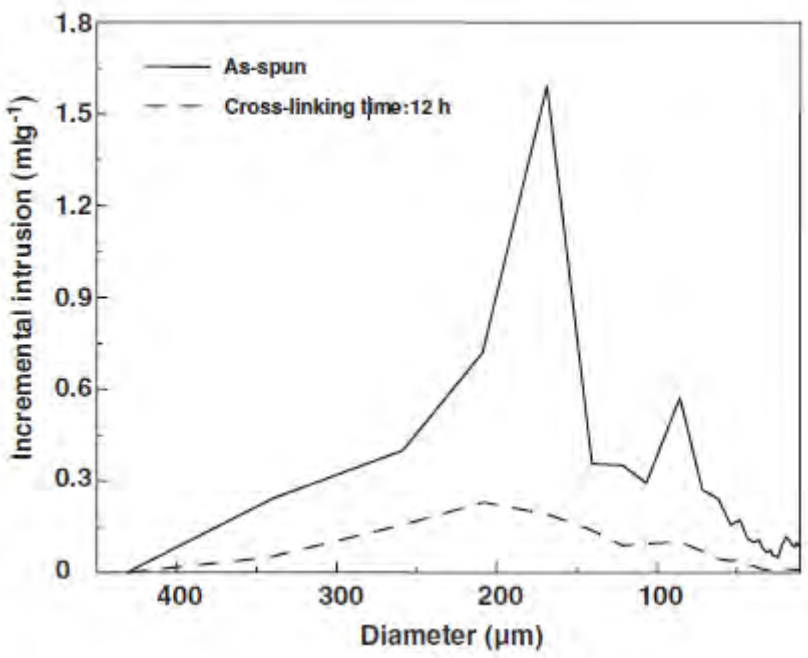


Fig. 6: Effect of vapor phase cross-linking on the pore size distribution of as-spun collagen matrix(37).

The maximum pore size of the as-spun collagen matrix was appropriately 180 µm, while that of the cross-linked collagen matrix was approximately 210 µm. However, the amount and distribution of pores in the cross-linked collagen matrix were smaller and narrower, respectively(37).

Mechanical Properties.

The mechanical properties of the cross-linked collagen nanofibrous matrices were assessed using a tensile test. The maximum load value of Instron® (www.instron.it) on the collagen matrix was evaluated. Table 2 shows that the tensile strength of the collagen matrix (0.2mm thick) was above 10MPa(37).

Table 2: Tensile strength of the cross-linked collagen nanofibrous matrix and of three commercial matrices. Values in parentheses represent the thickness of matrices. Data are expressed as mean ±SD from three independent determinations. ANOVA[6]: P<0.05. Pairwise comparisons: *P<0.05 versus collagen NF.

Groups	Tensile strength (MPa)	Reference
Collagen NF (0.2 mm)[7]	11.44±1.20	
Collagen NF, hydrated form	7.40±1.17*	
Beschitin® W (1 mm)	15.89±0.63*	
Resolut® LT	11.72	(39)
Biofix®	14.50	(39)

The tensile strength of the collagen nanofibrous matrix was comparable to that of two commercial tissue regenerative membranes (Resolut® LT, Biofix®)(40) and a wound-dressing material (Beschitin® W; Unitika www.unitika.co.jp). Although the cross-linked collagen nanofibrous matrix had a lower tensile strength after being hydrated with phosphate buffer (pH 7.4), which is common for cross-linked hydrophilic

[6] ANOVA, analisys of variance.
[7] Collagen NF, cross-linked collagen nanofibrous matrix.

materials, the matrix presented enough stability to maintain its shape for in vivo procedure. Therefore, there is no mechanical problem in using a collagen nanomembrane in vivo as the wound-dressing material. As the collagen nanofibrous matrix possesses almost identical tensile strength to commercial products, it was anticipated that the collagen nanofibrous matrix could be a suitable tissue engineering scaffolding for skin regeneration(37).

Cross-linking technologies.

The collagen nanofibrous structure can be changed into a dense membrane structure by cross-linking in an aqueous system, even though the aqueous medium contains a small amount (below 5%) of water. Therefore, vapor cross-linking by glutaraldehyde can be chosen to avoid the collapse of the collagen nanofibrous matrix during cross-linking in the aqueous system. Additionally, the unreacted aldehyde groups are treated with 0.1 N glycine aqueous solution after vapor cross-linking. Fig. 7 shows the effect of cross-linking time on the weight loss (%) of the collagen nanofibrous matrix(37).

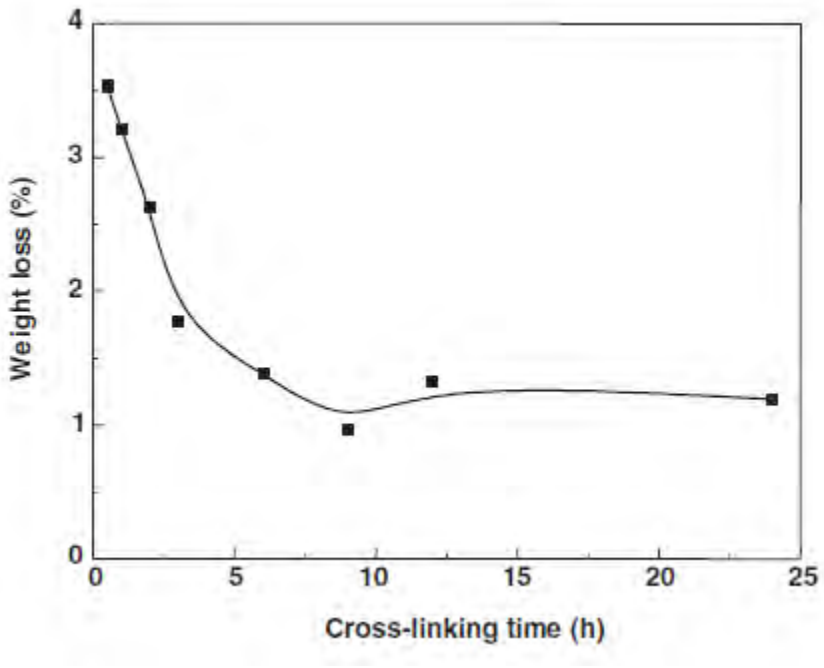


Fig. 7: Weight loss of the collagen nanofibrous matrix by cross-linking time(37).

The weight loss (%) of collagen matrix was determined by weighing the dried sample before and after the immersion in distilled water for 1 h. The as-spun (uncross-linked) collagen matrix showed a weight loss of 3.6% and was highly swollen in water. As cross-linking time increased up to 9 h, the weight loss values of collagen matrices gradually decreased and they swelled less in water. Considering the weight loss and dimensional stability of cross-linked collagen matrices, we choose vapor cross-linking for 12 h as an appropriate condition. Fig. 8 shows a SEM micrograph of a collagen nanofibrous matrix after cross-linking for 12 h(37).

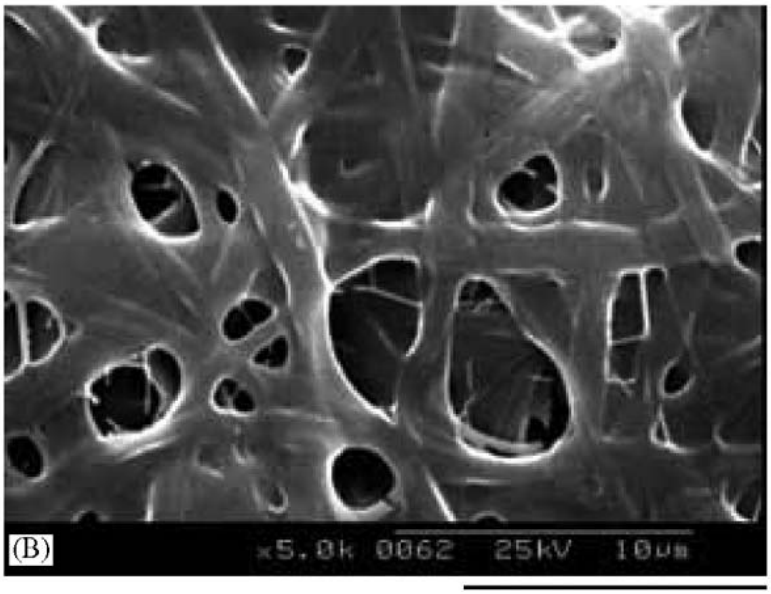


Fig. 8: SEM images of collagen nanofibrous matrices after vapor phase cross-linking for 12 h. Bar, 10 µm; 5000x magnification(37).

Cell behaviour, tissue regeneration, ligands.

Effects on cytocompatibility, cell behavior, cell and collagen nanofiber interactions, and open wound healing in rats were examined. Relatively low cell adhesion is observed on uncoated collagen nanofibers, whereas collagen nanofibrous matrices treated with type I collagen or laminin are functionally active in responses in normal human keratinocytes. Collagen nanofibrous matrices are very effective as wound-healing accelerators in early-stage wound healing(37).

ECM proteins, such as type I and type IV collagen, laminin, fibronectin, and vitronectin, are effective in promoting cellular adhesion and spreading. These proteins possess a arginine–glycine–aspartic (RGD) acid sequence, that is recognized by integrins(41). Collagen binds mainly to the integrins α1β1 and α2β1 and affects the attachment and differentiation of osteoblastic cells(42).

Nonwovens of electrospun collagen nanofibers were cut out with punch (14-mm in diameter) and put onto 24-well culture plates (Nunc, Denmark). The 24-well culture plates containing collagen nanofibers were coated with 200 µl/well of ECM proteins, in this case type I collagen (50 µg/ml), fibronectin (1 µg/ml), or laminin (10 µg/ml) in phosphate-buffered saline (PBS), by overnight adsorption at 4°C. The effect of 0.1–50 µg/ml of type I collagen, fibronectin, and laminin was tested on human oral keratinocyte adhesion and spreading to the culture plate surface and found that 50 µg/ml type I collagen, 1 µg/ml fibronectin, and 10 µg/ml laminin showed the approximately maximal effect on NHOK (data not shown). The wells were then washed with PBS and unbound sites were blocked with PBS containing 1% heat-inactivated bovine serum albumin (BSA). The plates were rinsed again with PBS. Cells were detached by treatment with 0.05% trypsin and 0.53mM ethylenediaminetetraacetic acid (EDTA) in PBS, resuspended in the culture media ($1x10^5$ cells/500 µl), added to each plate, and incubated for 1 h at 37 1C. Unattached cells were removed by rinsing twice with PBS. Attached cells were fixed with 10% formalin in PBS for 15 min and rinsed twice with PBS. Cells attached to the collagen nanofibers were stained with hematoxylin and eosin. The wells were gently rinsed three times with double distilled water (DDW). The electrospun collagen nanofibers were mounted, and cells attached onto the nanofibers were photographed. To ensure a representative count, each nanofiber sample was divided into quarters, and two fields per quarter were photographed with an Olympus BX51 microscope at 100x. Cell spreading was analyzed using photographs of the cell adhesion

assay. To ensure a representative count, each nanofiber sample was divided into quarters, and two fields per quarter were photographed with an Olympus BX51 microscope at 100x. Cells that adopted a flattened, polygonal shape with filopodia- and lamellipodia-like extensions were regarded as spreading cells. In contrast, cells that resisted washing and remained tethered to the plate surface were regarded as nonspreading cells. The percentage of cells displaying spread morphology was quantified by dividing the number of spread cells by the total number of bound cells(37).

The absence of cytotoxicity of collagen had already been demonstrated(32).

Cross-linked collagen nanofibrous matrices were seeded with normal human keratinocytes, such as NHOK and NHEK. The adhesion of cultured keratinocytes was evaluated using a cell adhesion assay in serum-free medium. Exponentially proliferating NHOK[8] and NHEK[9] adherent to the electrospun collagen nanofibers were microphotographed in the adhesion assay after washing, fixing, and staining the cells with hematoxylin and eosin. Unexpectedly, a relatively low level of cell adhesion of normal human keratinocytes was observed on collagen nanofibers without ECM coating compared to a polystyrene surface. Although the reason for this low adhesion remains unknown, it may be a consequence of denaturalization of the collagen conformation induced by electrospinning or cross-linking. Generally, the procedures used to reprocess a natural protein into an engineered material compromise many of its biological and structural properties(43).

The adhesion of cells on collagen nanofibrous matrices treated with ECM proteins was examined for their ability to restore collagen's biological and structural properties. The adhesion of cultured keratinocytes was evaluated with a cell adhesion assay in serum-free medium, using type I collagen (50 µg/ml), fibronectin (1 µg/ml), or laminin (10 µg/ml), which were adsorbed onto the collagen nanofibers as substrates. Type I collagen and laminin significantly promoted the adhesion of proliferating NHOK and NHEK compared with collagen nanofibers only (Fig. 9 and Fig. 10).

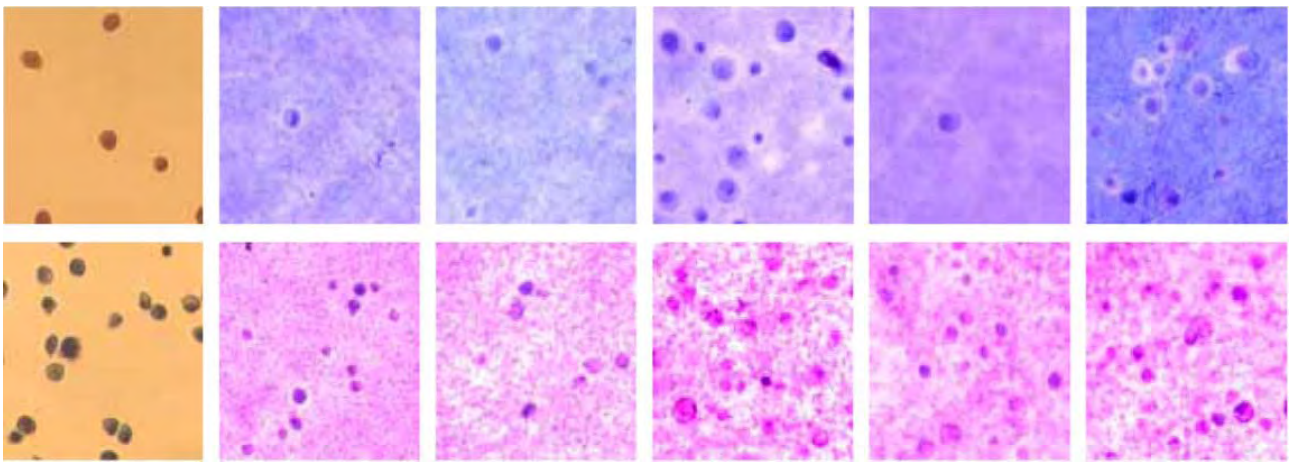


Fig. 9: Examples of cell adhesion and spreading on type I collagen, fibronectin, or laminin in proliferating NHOK and NHEK(37).

[8] NHOK, normal human oral keratinocytes. NHOK are isolated from human gingival tissue specimens obtained from healthy volunteers (age range 20–30 years) undergoing oral surgery. Oral keratinocytes are isolated from separated epithelial tissue by trypsinization, and primary cultures are established in a keratinocyte growth medium containing 0.15mM calcium and a supplementary growth-factor bullet kit (KGM; Clonetics, San Diego, CA) (37).

[9] NHEK, normal human epidermal keratinocytes. NHEK, prepared in a manner similar to the NHOK, are obtained from human foreskins of patients (1–3 years of age) undergoing surgery. NHEK were also cultured in KGM (37).

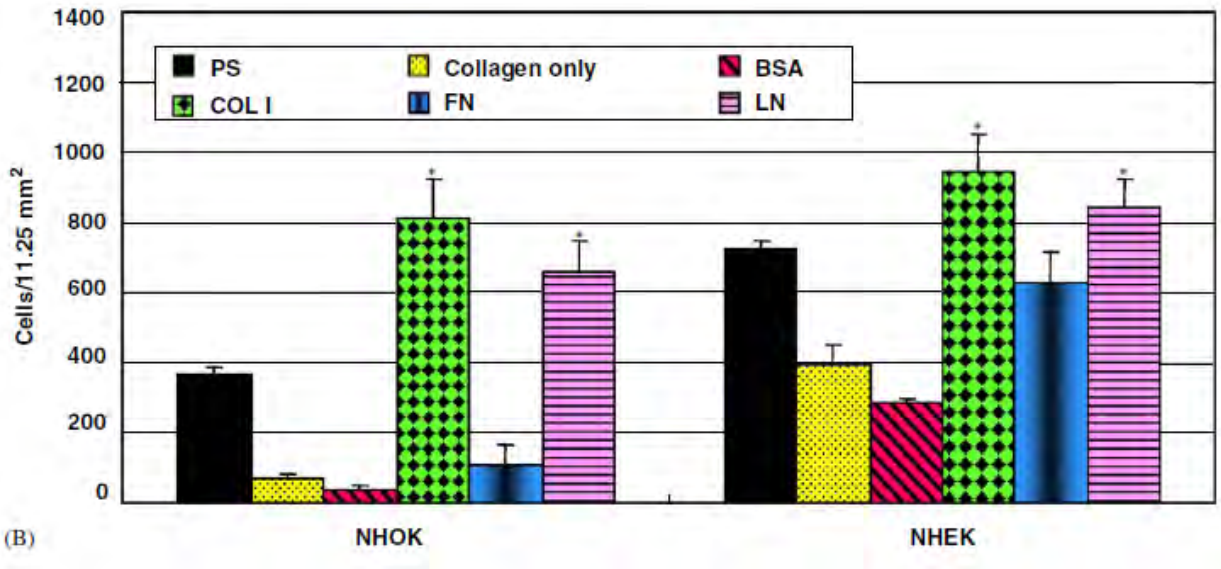


Fig. 10: Level of cell adhesion of cultured cells on ECM proteins(37).

The adhesion activity profile of fibronectin-coated electrospun collagen nanofibers on NHOK was similar to that of uncoated collagen nanofibers, but cell adhesion was slightly promoted in NHEK. These results indicate that type I collagen and laminin, which are integrin ligands, are functionally active in cell adhesion onto electrospun collagen nanofibers in normal human keratinocytes. To further evaluate the adhesion of type I collagen, fibronectin, and laminin, we determined whether adherent cells were tethered to the substrate or spread over the substrate. Photographs of exponentially proliferating NHOK and NHEK adherent to integrin ligands were taken in the adhesion assay. For type I collagen, 22% of the proliferating NHOK and 55% of the proliferating NHEK showed a spreading morphology (Fig. 11) i.e., they adopted a flattened, polygonal shape, with filopodia- and lamellipodia-like extensions. The remaining nonspreading cells on integrin ligands resisted washing and remained tethered to the nanofiber surface (data not shown). Laminin displayed functional properties similar to type I collagen, as 18% of proliferating NHOK and 47% of proliferating NHEK displayed a spreading morphology. In contrast, extremely low cell spreading was observed on either BSA- or fibronectincoated collagen nanofibers (Fig. 11). These results indicate that type I collagen is similar to laminin in that it supports cell adhesion and spreading, but that fibronectin is functionally inactive in terms of cell adhesion and spreading (37).

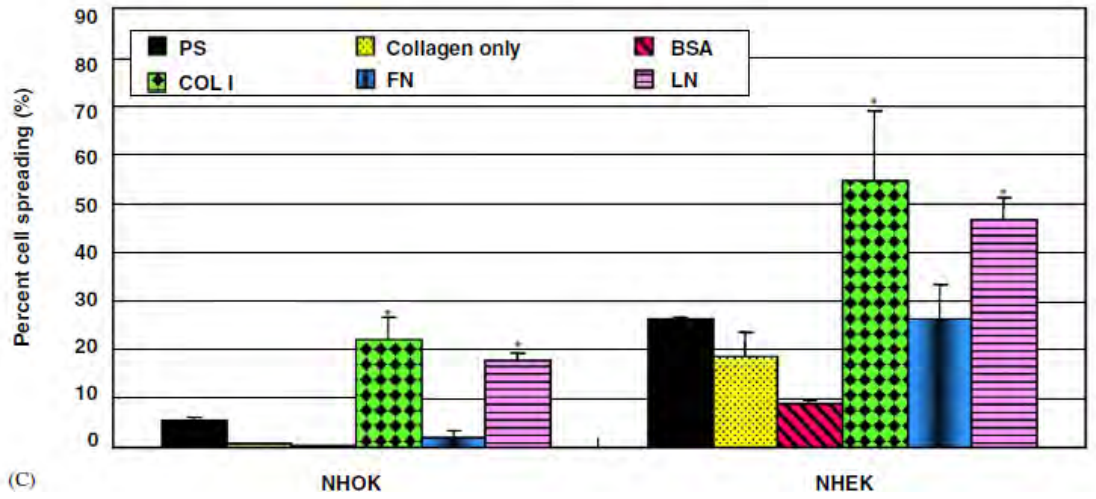


Fig. 11: Incidence of cultured cells spreading on ECM proteins(37).

In open wound healing tests, twin full-thickness rectangular wounds were made on the back of each rat. On the 7th and 10th days, wound closure in the collagen nanofiber-covered wounds was similar to the control group that had wounds covered with gauze (data not shown). In contrast, microscopic examination revealed that early-stage healing in the collagen nanofiber group was faster than that of the control group (Fig. 12). In the 1-week control group, the wound surface was covered by fibrinous tissue debris, and below that layer, dense infiltration of polymorphonuclear leukocytes and proliferation of fibroblasts were seen. In the 1-week collagen nanofiber group, however, the surface tissue debris disappeared, and there was prominent proliferation of young capillaries and fibroblasts. Late-stage healing processes in the control group were similar to that of the collagen nanofiber group (Fig. 12). In both groups, epithelization of the wound was complete after 4 weeks. Additionally, inflammatory cells disappeared and connective tissue was densely formed (37).

As described above, the fabrication of a collagen nanofibrous matrix via electrospinning and cross-linking appeared to provide good mechanical strength, even in aqueous solution. Furthermore, collagen nanofibrous matrices coated with type I collagen or laminin were functionally active in terms of cell attachment and spreading in normal human keratinocytes. Additionally, the electrospun collagen nanofiber nonwovens potentially provide a three-dimensional structure for cell attachment, growth, and migration. Therefore, cell morphology and the interaction between cells and collagen nanofibers, alone or in conjunction with an ECM protein coating, were studied in vitro for 7 days. SEM micrographs showed that NHOK adhered and spread on the surface of the collagen nanofibrous network and had started to migrate through the pores and to grow under layers of the fibers on day 7 (Fig. 13)(37). This is supported by another study that demonstrated that electrospun collagen nanofibers were densely populated with smooth muscle cells within 7 days and that smooth muscle cells were observed deep within the matrix and fully enmeshed within the fibrils of the electrospun collagen(32).

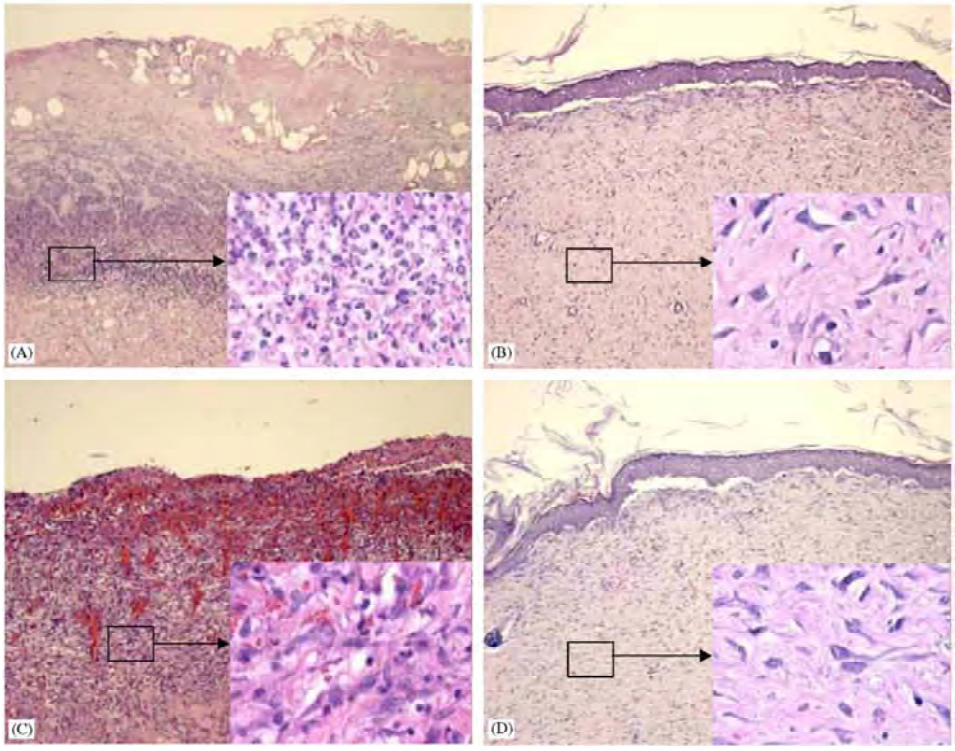


Fig. 12: Representative photomicrographs of wound healing of rat skin; control group at 1 week (A), control group at 4 weeks (B), collagen nanofiber group at 1 week (C), and collagen nanofiber group at 4 weeks (D) (H&E, 100). Insets: 400x magnification(37).

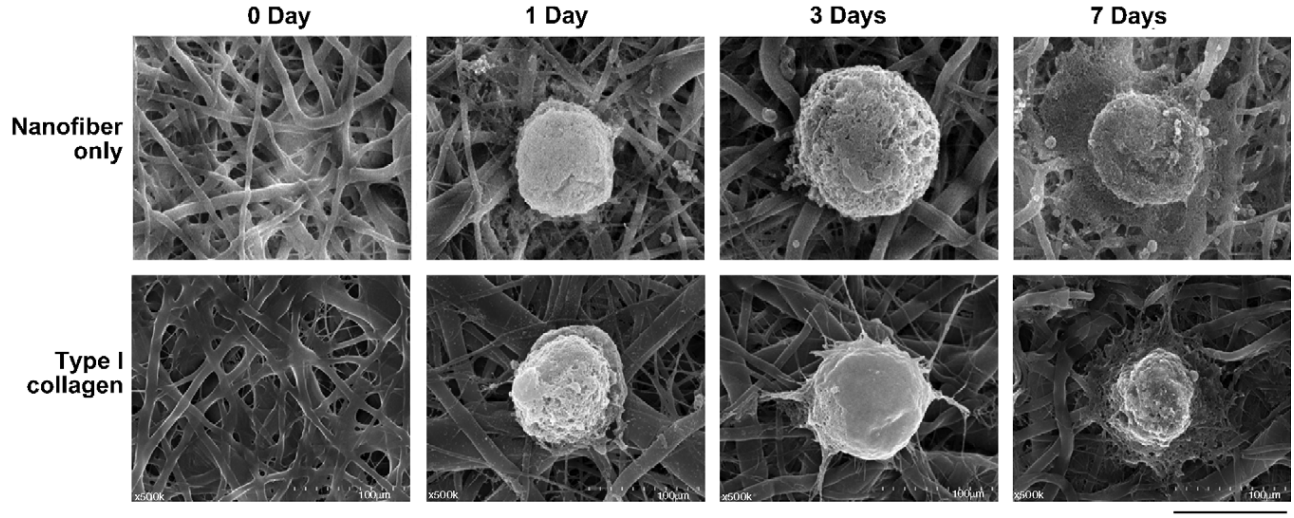


Fig. 13: SEM micrographs of the interaction between NHOK and an electrospun collagen nanofibrous structure coated with type I collagen after 0, 1, 3, and 7 days of culture. Bar, 10 µm(37).

However, type I collagen- and laminin-coatings on collagen nanofibers significantly promoted interactions between cultured oral keratinocytes and collagen nanofibrous matrices, in the following order: type I collagen4laminin4uncoated collagen nanofibers. NHOK also adhered and spread on the surface of the type I collagen- and laminin-coated collagen nanofibrous network and migrated through the pores and grew under layers of the fibers within 1–3 days. These cells interacted and integrated well with the surrounding fibers and grew in the direction of fiber orientation, forming a three-dimensional network of nanofibrous structure (Fig. 13). Observation is consistent with earlier reports demonstrating that electrospun biodegradable scaffolds are capable of supporting cell attachment and proliferation of human bonemarrow-derived mesenchymal stem cells(44) and normal human keratinocytes(45). These results support the idea that type I collagen and laminin, the integrin ligands, are functionally active in promoting cell adhesion and spreading of normal human epithelial cells onto the collagen nanofibrous matrices. Additionally, surface modification of biomaterials with ECM proteins or peptides was extensively tested. Given that the final goal of the scaffold design is the production of an ideal structure that can replace natural ECM proteins until host cells can repopulate and resynthesize a new natural matrix, cross-linked collagen nanofibers coated with ECM proteins, particularly type I collagen, may be a good candidate for biomedical applications, such as wound dressing and scaffolds for tissue engineering(37).

Electrospinning

Electrospinning has existed in the literature for more than 100 years(46). It was utilized to fabricate industrial and household nonwoven fabric products in the early 1930s(47). The technique has been rejuvenated recently as a very active research area because of its ability to produce fibers with diameters down to the sub-micron or nanometer range, significantly smaller than that can be achieved by other textile technologies such as melt spinning. The basic equipment setup includes a polymer solution (or melt) reservoir with a spinneret, an electric field (at a high voltage), and a grounded target collector. Under the electric field, the pulling force overcomes the surface tension of the polymer solution (or melt) and generates a charged jet that travels in a straight line for a certain distance. It then bends due to electrical instability to follow a whipping and spiraling path, reducing the jet diameter. The solvent evaporates on the way and the jet solidifies to form a non-woven fibrous mat on the grounded collector(48),(49). An electrically grounded rotating drum can be used as the collector to achieve preferred orientation if desired (Fig. 14) (32),(50).

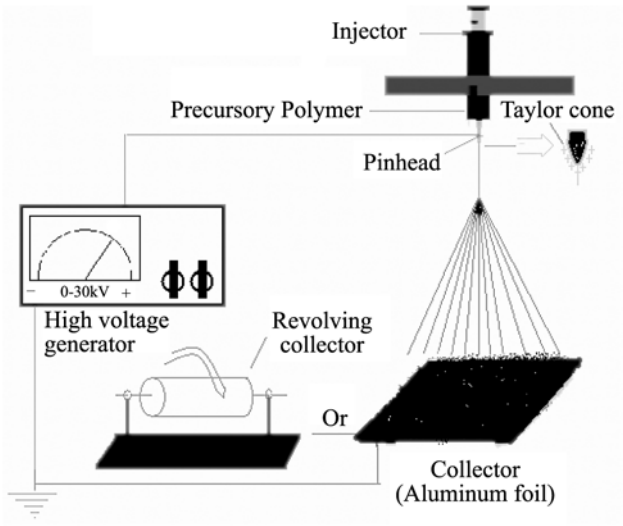


Fig. 14: Electrospinning scheme.

A variety of natural macromolecules (e.g. collagen, silk fibroin, and fibrinogen) and synthetic polymers (e.g. PGA, PLLA, PLGA, and PCL) have been processed into fine nonwoven fabrics for tissue engineering research(32),(51),(52). Various cells have been reported to attach, proliferate, and differentiate into or maintain their functional phenotypes on these electrospun nano-fibrous materials(32),(44),(53),(54),(55),(56). However, significant challenges still exist in using this technique to fabricate complex 3D scaffold shapes or to generate designed internal pore structures. In addition, the electrospun fibers using FDA approved biodegradable polymers (e.g. PLLA and PLGA) often have diameters in the micrometer range(57).

1.2. Scaffold main features

Several parameters affect the performance of a tissue engineered scaffold. Besides the biological factors, such as cell seeding density and nutrient concentration, the microstructure of the scaffold itself plays an important role in cell attachment, penetration depth and tissue vascularization (58).

Several studies have demonstrated that cell growth into scaffolds depends upon to which extent nutrients can permeate through the porous structure during the cell culture process (59)(60)(61)(62). Increased nutrient flux across scaffolds yields better cell culturing results. On the other hand, nutrient flux across scaffolds is strictly related to scaffold permeability.

The permeability of a scaffold is determined by a combination of micro and nanostructural factors including porosity, pore size, geometry and distribution, pore interconnectivity, fenestration size, and the orientation of pores with respect to flow direction (59).

Permeability is independent of the fluid and the thickness of the porous medium. In fact, it quantifies the ability of a porous medium to transmit fluids through its interconnected pores or channels when subjected to pressure.

The permeability of biological tissues, and of tissue-engineered scaffolds as well, plays a primary role in the transport of nutrients and metabolites through cellular shells, driven by diffusion and/or advection through capillary networks formed throughout the scaffold via angiogenesis (63). The influence of fluid flow has been studied in a variety of tissues (64)(65)(66)(67). Furthermore, scaffold permeability also influences cyclic changes in biophysical stimuli due to the fluid flowing through the structure during mechanical

loading. Specifically, permeability affects the magnitude of pressure and fluid shear stresses within the construct or tissue and they have been identified as potential stimuli for cellular differentiation or functional adaptation (68)(69)(70). Construct permeability has also been shown to influence the degradation rate of biodegradable scaffolds for TE (71). Additionally, construct chemical composition can influence permeability. Different biological compounds such as glycosaminoglycans and collagen (72) have been shown to affect the permeability of many different tissues.

Many permeability measurement systems have been proposed, depending on their application. A gas permeability measurement system was developed for partially saturated geosynthetic clay liners (GCL) for waste containment facilities, where nitrogen was used to flow through the porous material (73). Pressure gauges and a gas flow meter were used to measure the induced pressure drop and the gas flow rate. An instrument to measure fabric air permeability at high pressure levels was constructed (74). A tank of sufficient capacity precharged to a suitable pressure level was used to supply air to the fabric. A pressure sensor was used to monitor the air pressure of the tank. The permeability of apple tissue (75) was measured by an apparatus in which manometers and a flow meter were used to measure the induced pressure and the flow rate. Using linseed oil to flow through a bone sample by gravity-induced pressure, human trabecular bones were measured (62). The permeability of the bone sample was determined based on the amount of the linseed oil flowing through the sample within a certain period of time. A gravity-driven system was also used (76) to measure the permeability of ceramic-based foam using water as the percolating fluid. The induced pressure was calculated using the height of the water pipe and water fluid properties.

In general, permeability measurement systems suffer from several limitations when applied to tissue engineering. First of all, most of these systems use either water or oil as the fluid medium. Such systems require either a high induced pressure to force the fluid though or a long measurement time to allow the fluid to seep through. High induced pressure can deform porous scaffolds for soft tissue engineering and thus cause false readings. Long measurement time could cause complete saturation, which leave residual fluid in the sample and cause inaccurate results. Long measurement time is also a concern when water is used to measure biodegradable scaffolds, since the hydrolysis reaction can cause changes in the scaffold itself. Furthermore, surface tension of water or oil could block small pores of the porous sample, causing lower permeability readings (58).

In this framework it is clear that an ideal system for permeability measurement should guarantee from the achievement of rapid measurement operation without introducing any effect to the porous microstructure.

1.2.1. Parameters affecting scaffold performance

Among the structural parameters characterizing a scaffold, porosity and pore size are the primary factors for *in vivo* tissue ingrowth and reorganisation, as they directly affect the physiological diffusion of nutrients and waste products of cells metabolism. The pore size of the scaffold dictates the type of cell which will penetrate and proliferate within the structure (77).

The internal geometry of a porous medium is microscopically characterized by partitioning the pore space into a discrete and well-defined collection of individual pores. With this commonly-used conceptual model, pores can be rigorously defined as regions of the void space confined by solid surfaces (termed as nodal pores, pore bodies, chambers) connected by pore channels (termed as pore necks, throats) (78).

Porosity ε is defined as:

$$\varepsilon = \frac{V_V}{V_T}$$ **Eqn. 1**

where V_V is the volume of void space and V_T is the bulk volume (78).

The **effective porosity**, ε_e, also called the kinematic porosity, of a porous medium is defined as the ratio of the part of the pore volume where the water can circulate to the total volume of a representative sample of the medium.

In a saturated system composed of two phases (solid and liquid) where V_s is the volume of the solid phase, $V_w = (V_{iw} + V_{mw})$ is the volume of the liquid phase, V_{iw} is the volume of immobile pores containing the water adsorbed onto the soil particle surfaces and the water in the dead-end pores, V_{mw} is the volume of the mobile pores containing water that is free to move through the saturated system, and $V_t = (V_s + V_{iw} + V_{mw})$ is the total volume, the effective porosity can be defined as follows (79):

$$\varepsilon_e = \frac{V_{mw}}{V_T} = \frac{V_{mw}}{V_S + V_{iw} + V_{mw}}$$ **Eqn. 2**

The **pore size** is approximated by a distribution that can be expressed as (80):

$$s_p(r) = \frac{\alpha_p}{\sqrt{\frac{\pi}{2\sigma_p}}} e^{\left(-2\left(\frac{r-r_p}{\sigma_p}\right)^2\right)}$$ **Eqn. 3**

where α_p is the relative proportion of the distribution (that represents the sum of every pore radius) and σ its standard deviation, r is the radius of a sphere inscripted in a sphere-like porous or the radius of a cylinder inscripted in a channel-like porous, r_p the mean value of r. Eqn. 3 is just an interpolation of a stochastic phenomenon, whose direct measurement takes to a result like the one reported in Fig. 15.

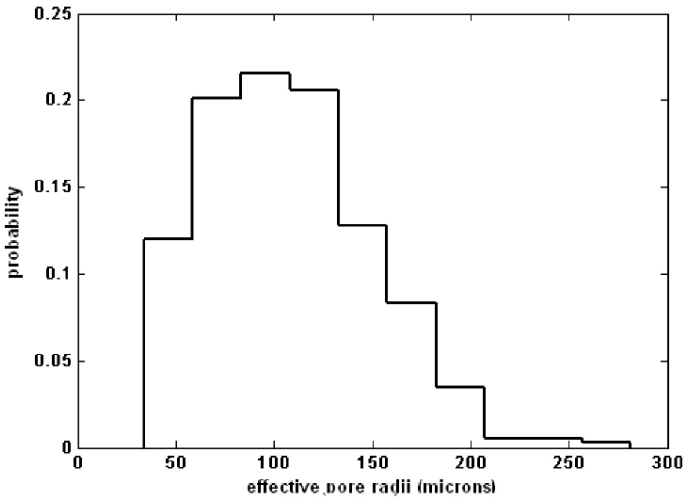


Fig. 15: Distribution of effective pore radii in a stochastic scaffold. representative stochastic scaffold (78).

Theoretical methods can be applied in evaluating scaffold porosity. The two main remarkable theoretical approaches are the so called “unit cube analysis” and the “mass technique” (81). The first method is commonly adopted for regular honeycombed scaffolds (Fig. 16). In the unit cube method the volume sum of the scaffold material and the associated pore spaces is calculated by taking linear measurements of the scaffold cube. This method is based on the assumption that the scaffold structures are maintained uniform during the manufacture. The volume of the scaffold material is calculated from a known deposition pattern, so these methods fail (*i*) when applied for calculation of scaffolds with irregular geometry/architecture; (*ii*) in the case of scaffolds manufactured by means of extrusion techniques.

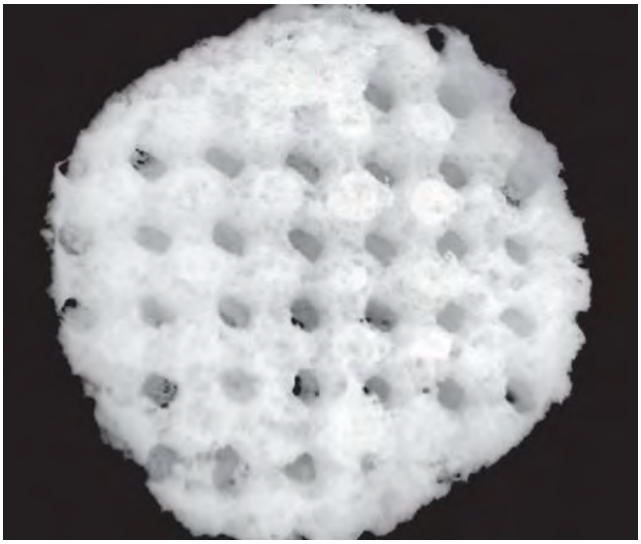


Fig. 16: Salt leaching fabricated globally and locally porous PLA scaffold (82)

In the mass technique, the scaffold is shaped as a cube. The volume of the scaffold material is derived by dividing the mass of the scaffold by its material density. The “apparent” scaffold volume is calculated from its linear measurements and scaffold porosity is derived. This method can be applied to scaffolds with both controlled and uncontrolled geometries, but its accuracy is strongly dependent on the linear measurements of the scaffold.

SEM (scanning electron microscopy) analysis complements these theoretical calculations of porosity and it allows direct measurements of pore size and strut/wall thickness (83) (84).

Mercury porosimetry is a technique that employs mercury which is a non-wetting liquid that does not intrude into pore spaces except under sufficient pressure. In this technique, the scaffold is subjected to gas evacuation while allowing mercury to flow. Pressure is applied and the sample is enveloped by mercury. From the mercury volume measurements, the bulk volume of the sample is derived. Bulk volume is equivalent to apparent volume and it consists of scaffold material and pore spaces. Maximum pressure is attained through incremental steps so as to promote mercury intrusion. At maximum pressure, the total volume of the intruded mercury is measured, and this allows the derivation of total open pore volume and porosity. Using the Washburn equation (85), pore sizes and pore volume distribution by pore size are calculated (86). Pore sizes smaller than 0.0018 μm are not intruded with mercury and this is a source of error for porosity calculations. Furthermore, mercury porosimetry does not account for closed pores as mercury does not intrude into closed pores (87). Because of the high pressure applied by the device (until 400 MPa), mercury porosimetry is not suitable for fragile compressible scaffolds such as flexible foams (with porosities higher than 90%), textiles and nanofiber sheets. Other concerns would include the fact that the Washburn equation assumes prefect cylindrical pores, which is not the case in reality.

Gas pycnometry is capable of measuring scaffold porosity without the need to measure the scaffold material volume (88) (89), employing a gas displacement method. This technique operates by detecting the change in pressure when the scaffold specimen displaces gas: a specimen of unknown volume is placed in the sample chamber of known volume; upon sealing, the chamber pressure is measured. A reference chamber, with known volume and pressure, is separated from the sample chamber by a closed valve. Both systems are allowed to equilibrate to a pressure when the valve is opened. Using gas law, the unknown volume of the specimen is obtained (90). Once the scaffold material volume is determined, using the cube approach allows the determination of the scaffold porosity. There are typically two sources of error in this volumetric measurement, firstly it does not take into account the closed pores which the gas molecules cannot enter. Secondly the reliability of this method is hampered by the difficulty in taking accurate linear dimensions of the unit scaffold cube.

In solids, electrical forces of attraction hold down the atoms in their equilibrium positions. As the outermost atoms have lesser neighbours than those beneath them, an imbalance of attractive forces occurs. As a consequence, in order to maintain the equilibrium, surface atoms attract surrounding gas molecules via Van der Waals and electrical forces. This physical phenomenon is known as gas adsorption and it can be applied in the study of porous materials (91) (92) (93). The gas adsorption procedure begins with the placement of the specimen in an evacuated chamber where a small amount of adsorbate gas (nitrogen, benzene vapour, argon and krypton) is introduced. Adsorption isotherms are derived from the pressure and volume measurements of the chamber. First, adsorbate molecules form an initial thin layer on the available surfaces: at this stage, the surface area can be calculated using the well consolidated Brunauer, Emmett and Teller (BET) theory (94) and the Langmuir model (95) (96) (97). As gas adsorption continues, multi adsorption layers form and capillary condensation occurs: at this stage, pore sizes can be derived using the Barnett, Joyner and Halenda (BJH) method. When the pores are completely saturated with adsorbate molecules, the total pore volume of the scaffold is calculated. Gas adsorption setups are capable of assessing scaffolds with pore sizes ranging from 0.35 to 400 nm or 3.5 to 2000 mm. Setups capable of small pore size measurements can be used to evaluate the architectural parameters of nanofibers, dense foams and textiles. However, gas adsorption is not a suitable approach in studying scaffolds with low specific surface area, as erroneous surface area measurements would be encountered for values lesser than 0.01 m^2/g and closed pores do not allow the entry of adsorbate molecules, thus gas adsorption fails to account for the presence of these pores (81).

Flow porosimetry is a non-destructive approach that has been used to measure the pore sizes of porous materials (81). In this technique, a fully wetted scaffold sample is sealed in a chamber and gas is allowed to pass through it. At the bubble point, there is sufficient gas pressure to overcome the fluid capillary action in the largest pore. The pressure is increased while the flow rate is measured till all the scaffold pores are empty and dry. Pore size distributions and mean pore size can be derived once the flow rate and the applied pressure are known. Flow porosimetry is capable of measuring pore sizes within the range of 0.013–500 μm. Furthermore, compressive stresses can be applied onto the sample so as to study the effects of compression on pore size and distribution. Flow porosimetry cannot measure the overall porosity.

Given the complex geometry of random porous media in general and stochastic TE scaffolds in particular, the microscopic and macroscopic geometric features can be investigated from the definition of a *skeleton*, i.e the medial axis representation of the porous space. A skeleton can be built identifying the centres of each pore, defined *nodes*, connecting them, when possible, by simply connected paths, called *links*, and fusing them in a whole geometric entity (Fig. 17).

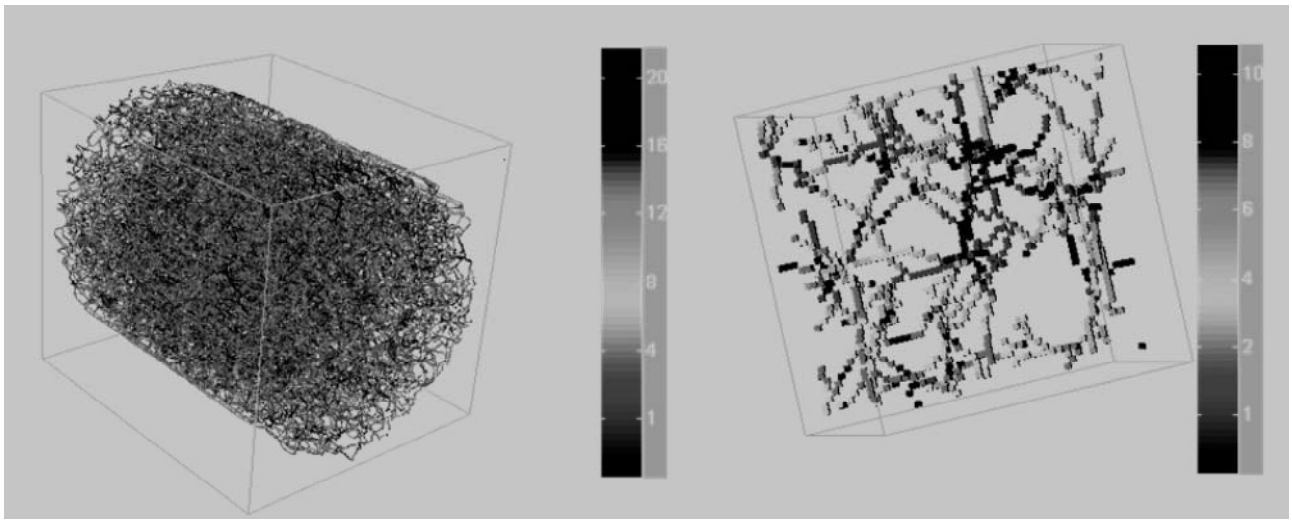


Fig. 17: Qualitative visualization of the skeleton of a scaffold. The left panel shows the medial axis for the complete scaffold; the right panel shows the medial axis for a central subvolume (78).

The skeleton has a strict geometrical relationship to the object's surface and preserves the salient geometric properties of the object.

Having partitioned the skeleton of the pore space into links and nodes, the pore space **interconnectivity** is determined by finding the distribution of coordination number c, i.e. the number of pore throats associated with a pore body. To establish a one-to-one correspondence with the pore and the throat, adjacent throats are coalesced (78). The distribution can be expressed

$$i(c) = \frac{\propto_c}{\sqrt{\frac{\pi}{2\sigma_c}}} e^{\left(-2\left(\frac{c-c_m}{\sigma_c}\right)^2\right)} \qquad \textbf{Eqn. 4}$$

where $\propto_c$ is the relative proportion of the distribution (that represents the full amount of links in the scaffold), σ_c its standard deviation and c_m the mean value of c (80).

Fenestration size is defined as the value of the mean radius of the throats, calculated over a link. For each point of the link, the local radius is evaluated on the plane orthogonal to the link itself. Even for the fenestration size, a distribution can be defined as for porosity and interconnectivity (Eqn. 3 and Eqn. 4) (78).

Geometrical **tortuosity** is defined as the distribution of the shortest path lengths through the skeleton between two nodes (78). Given two nodes i and j, the shortest path s_{ij} (if it exists) from $i$to j through the skeleton is determined along with the Euclidean distance d_{ij} between i and j. The geometrical tortuosity T_{ij} of the path is defined as

$$\mathrm{T}_{ij} = \frac{s_{ij}}{d_{ij}} \qquad \textbf{Eqn. 5}$$

Geometrical tortuosities are computed for the shortest paths between all possible skeletal nodes (78). Anyway, an average tortuosity *T*, mean value of Eqn. 5, can be effectively defined to be used in calculation.

For isotropic porous medium, Bear and Bachmat (98) argued that the effective viscosity is related to the porosity through the following relation:

$$\frac{\tilde{\mu}}{\mu} = \frac{1}{\varepsilon} T \qquad \text{Eqn. 6}$$

where ε and T are the porosity and tortuosity of the medium, respectively.

Unfortunately, the parameters considered till this point are inadequate and have led to confusions when it comes to relating them to the biological performance of TE scaffolds (59). For instance, scaffolds with high porosity were expected to perform better than those with lower porosity, but it was demonstrated (99) that highly porous scaffolds do not always encourage cell proliferation, differentiation and reorganisation. In scaffolds with uncontrolled micro-architecture, for instance, pore size can result in non-uniform cell density within the scaffold: occlusion may also occur at smaller pore sizes, thus preventing cellular penetration and matrix formation (100). In addition, some of the conventional parameters are hard to assess. For example, tortuosity can be an inaccurate characterization of the microstructure since there is no easy way of measuring the actual path lengths of the flow channels (101).

On the other hand, permeability allows a better evaluation of scaffold performance, it's easier to assess so it can be used as a reliable parameter.

1.2.2. Micro-CT scaffold image acquisition

Micro-computed tomography (micro-CT) is a "miniaturized" version of computerized axial tomography: by means of micro-CT a voxel resolution of the order of a few micrometers can be achieved. Up to now, the use of micro-CT has been successfully used in different branches of science for the study of porous or cavity-containing objects such as metallic foams, electronics, stones, wood and composite polymers. In particular micro-CT has been used to quantify complex geometries in 3D in great details: in bone biology, for instance, a great body of literature is concerned with the measurement of characteristics of the trabecular network (102). In the 1980s this technique has been extensively applied in the study of the 3D micro-architectural changes occurring in the various types of osteoporosis and the method has also provided interesting results in the survey of anti-osteoporotic treatments which can preserve bone architecture. More recently it has been applied in the study of cancer diseases as well as for the study of properties and characteristic of both natural and new-manufactured biomaterials. Fig. 18 displays the ability of this technique in micro-anatomical visualization of the micro-architecture of porous materials, and its potency as toolkit for 3D reconstruction.

By high resolution micro-CT, reconstruction and modelling of cancellous human bone sample has been achieved (Fig. 19).

On the basis of the previously collected data, characterization of the porosity of the commercial scaffolds has been carried out: dimensions (thickness) and distribution of solid particles, interconnecting pores and micro-cavities, have been extracted by means of an algorithm based on the direct distance transformation method (103), starting from micro-CT reconstructed models of the scaffolds (104). An example of the achieved results is shown in Fig. 20.

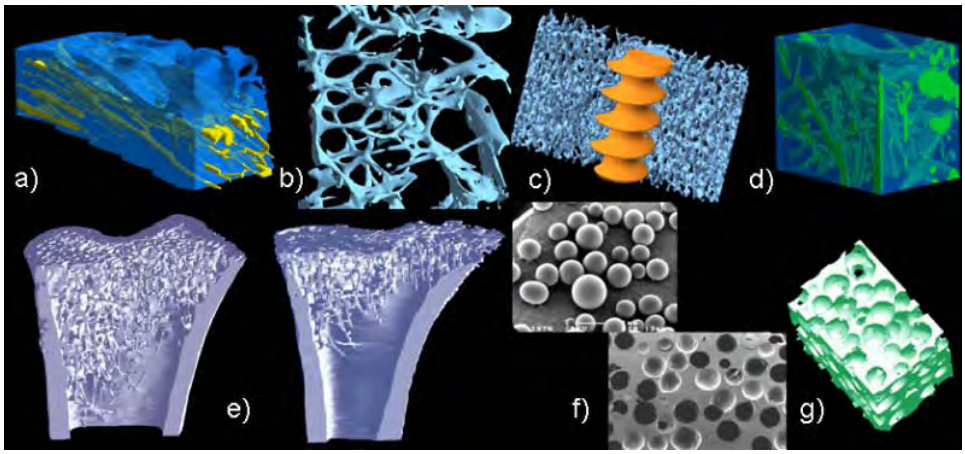


Fig. 18: 3D micro-CT reconstruction of a) the cortical bone (blue) with a reconstruction of the Haversian canals (yellow) to illustrate the complex branching of these structures; b) transiliac bone biopsy from a patient with glucocorticosteroid induced osteoporosis; c) a titanium surgical screw implanted in trabecular bone; d) overimposition of the porosity (green) through synthetic biomaterial (blue) composed of poly 2-hydroxymethacrylate with an interconnected porosity; e) tibial metaphysis of a control Wistar male rat and f) tibial metaphysis of an orchidectomised (ORX) rat 16 week after surgery, highlighting the importance reduction in bone mass and the marked conversion of trabeculae into pilars; g) SEM micrograph of polystyrene microbeads (left), porous poly 2-hydroxymethacrylate with porosity created by 1200 mm polystyrene microbeads (centre) and (right) the same porous block analyzed by micro-CT [http://www.med.univ-angers.fr/discipline/lab_histo/page_microCT.htm]

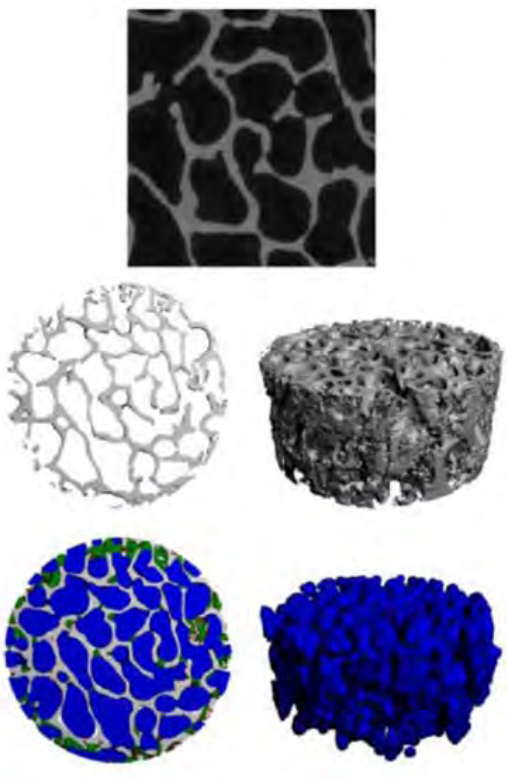


Fig. 19: a) detail of a cross-section obtained from a cancellous human bone sample; b) and c) 2D and 3D reconstructions of the cancellous human bone sample; d) and e) colour-encoded pore distribution (pores <60µm: red, pores 60–250µm green, pores > 250µm: blue) (104).

The generation and interpretation of micro-CT based aggregate pore models provides further insight into the expected osteoconduction and osteoinduction dynamics and therefore might serve as a basis for

further modifications of scaffold size and geometry as well as for further systematic investigations of the *in vivo/in situ* behaviour of bone substitute materials. A further application in scaffold modelling by micro-CT imaging methods has been reported (105), in which the porosity of biodegradable scaffolds manufactured by a selective leaching method for the porogenization of an ε-caprolactone-based scaffold was pre-determined (Fig. 21). In detail, using a well-defined multi-scale approach, the amount of

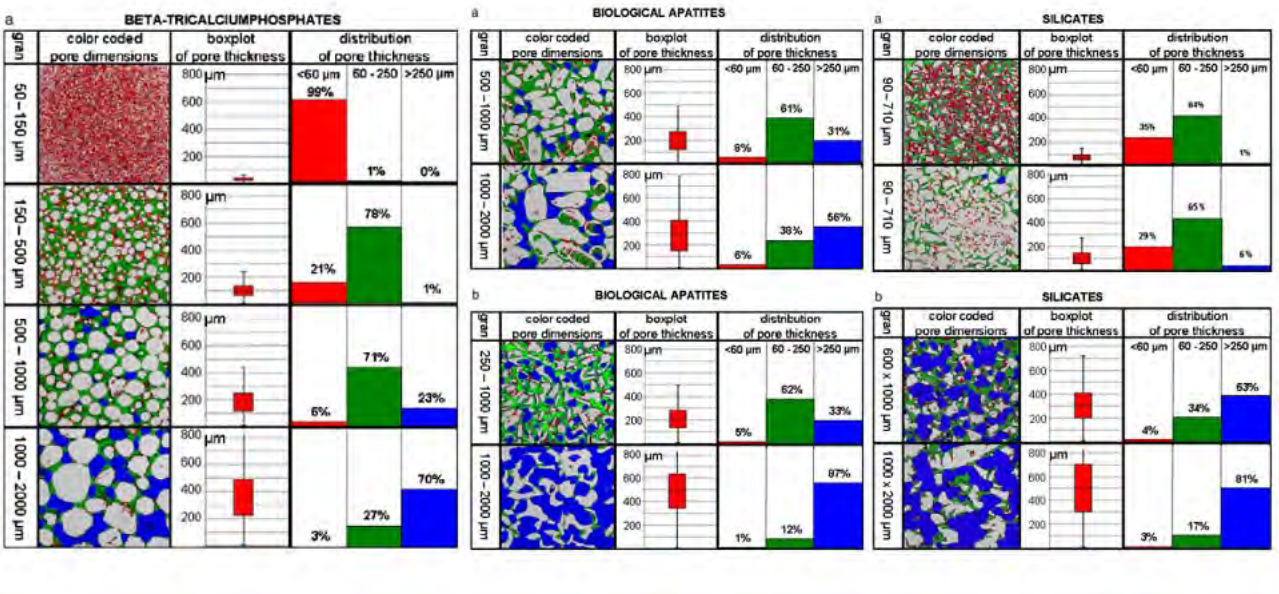


Fig. 20: characterization of porosity of different commercial materials for bone substituting. The described experimental methodology offers quantifiable information about 3D pore configurations (104).

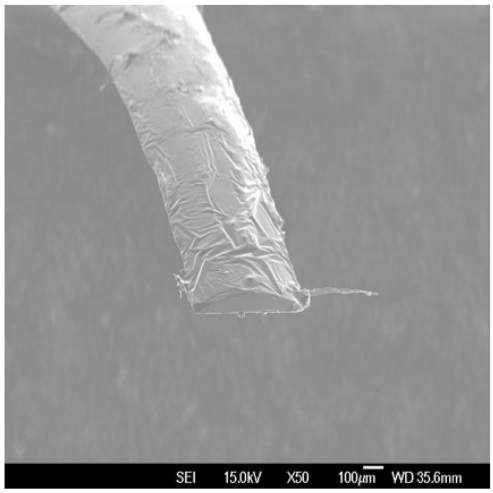


Fig. 21: SEM micrographs of porogen fiber (105).

the porosity following the leaching method was estimated and modelled by micro-CT analysis. In detail, a micro-CT system was used to grab images and on them quantify the 3D micro-structural morphology of the samples, at 16 μm resolution, as shown in Fig. 22. Micro-CT images identify the different pore structures obtained depending on the form of the porogen used. Interconnectivity of the scaffold pore network, estimated with cubic thickness model in micro-CT, confirms that the pores penetrated the device. In this case, micro-CT imaging has been successfully applied in the attempt of optimizing the requirements for the design of the scaffold.

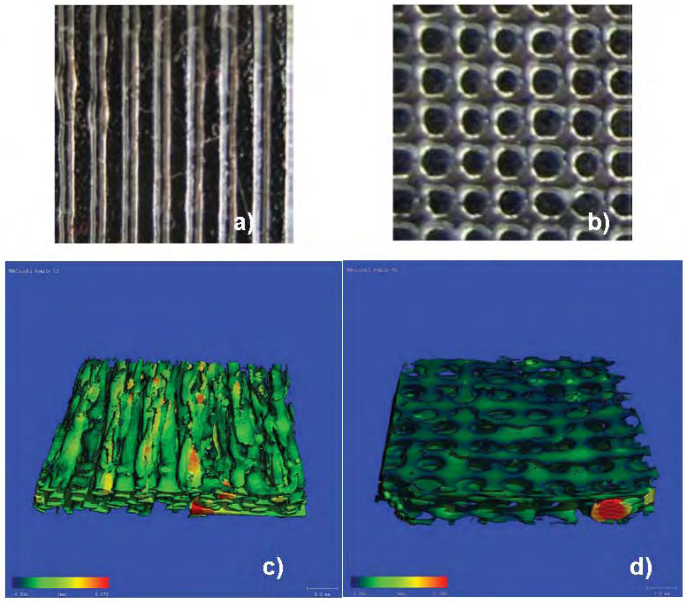


Fig. 22: 3D reconstructed visualizations of a) scaffold phase, b) scaffold with bone phase added (due to cellular ingrowth), and 3D network structure of the c) initial scaffold phase and d) after morphological rearrangement (due to cellular ingrowth) (105).

Imaging techniques have been also applied in the effort of analysing and characterising the microstructure of the manufactured scaffold during the cell growth and new tissue formation within the scaffold. 3D micro-CT imaging was used (106) for the assessment and the quantification of the 3D structure of a porous scaffold for bone ingrowth, aiming at visualizing the regenerative processes within the scaffold structure. The study was performed on porous bioceramic-based scaffolds, which have great potential in providing solutions for bone replacement. Even though micro-CT technique cannot provide all the data like a standard histological analysis, this technique has substantial potential advantages in identifying scaffold characteristics and bone ingrowth parameters in 3D (81) (107). Indeed, micro-CT imaging could suffer from limitations since insufficient resolution and inaccurate phase identification within the scaffold volume. This is due to the fact that a quantitative characterization of the initiation, onset and continuing growth of mineralized tissue within porous specimens (exhibiting pore sizes of 50–300 μm) requires imaging with resolutions of the order of at least 10 μm: only imaging at this resolution allows to accurately map pore size and structure, to quantify bone ingrowth phase fractions within individual pores and to study transport properties within the porous structure. Anyway, by means of micro-CT imaging, images with voxel sizes less than 1.5 μm are potentially achievable[10], making it superior to other techniques such as ultrasound (30 μm) and magnetic resonance imaging (100 μm). Moreover, the complex process of bone remodelling inside a tissue-engineered construct, made up of scaffold material, host bone, mineralized bone and soft tissue, makes the partitioning of the tomogram into discrete phases non-trivial. In the attempt to settle the previous issues associated with the use of micro-CT, a highly sophisticated three-phase segmentation approach was implemented (106) to develop a practical phase separation of the tissue-engineered construct with minimal artifacts. In particular, the developed imaging technique has resulted in analyzing and quantifying the following scaffold properties: *i)* porosity, *ii)* bone ingrowth volume, *iii)* pore volume, and *iv)* fraction of pore occupied by bone, furnishing a detailed description of the changes in scaffold morphology over time during the regenerative process of the bone within the scaffold (Fig. 23). This

[10] Value supplied by Xradia Inc.

example illustrates the potential of the micro-CT-based imaging techniques combined with advanced segmentation algorithms, in bone ingrowth assessment. The 3D visualization in Fig. 23 shows the presence of a relevant extent of the ingrowth through the centre of the scaffold.

Finally, micro-CT-based imaging joined with modelling techniques appear to be extremely promising tools to be applied not only in computer-aided scaffold designing but, in a non-destructive evaluation of the modification in scaffold morphology and characteristics during *in vivo*/*in vitro* new-tissue formation as well.

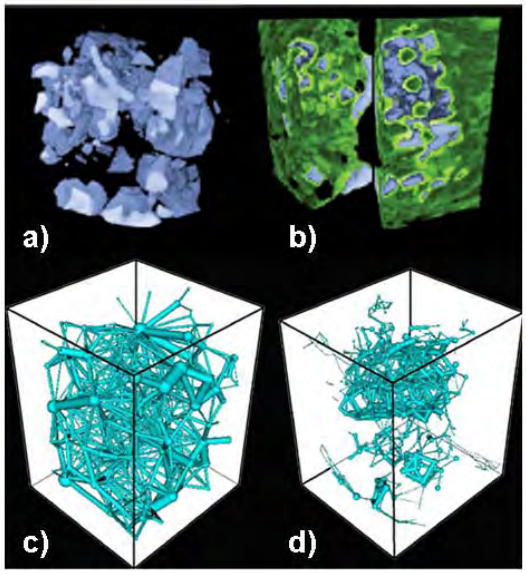


Fig. 23: photograph of a) paralleled fiber porogens and b) porogen mesh; micro-CT image of c) the pore structure within the matrix porogenized with paralleled fibers and d) the pore structure within the matrix porogenized with porogen mesh (106).

Many permeability measurement systems have been proposed, depending on their application. A gas permeability measurement system was developed for partially saturated geosynthetic clay liners (GCL) for waste containment facilities, where nitrogen was used to flow through the porous material (73). Pressure gauges and a gas flow meter were used to measure the induced pressure drop and the gas flow rate. An instrument to measure fabric air permeability at high pressure levels was constructed (74). A tank of sufficient capacity precharged to a suitable pressure level was used to supply air to the fabric. A pressure sensor was used to monitor the air pressure of the tank. The permeability of apple tissue (75) was measured by an apparatus in which manometers and a flow meter were used to measure the induced pressure and the flow rate. Using linseed oil to flow through a bone sample by gravity-induced pressure, human trabecular bones were measured (62). The permeability of the bone sample was determined based on the amount of the linseed oil flowing through the sample within a certain period of time. A gravity-driven system was also used (76) to measure the permeability of ceramic-based foam using water as the percolating fluid. The induced pressure was calculated using the height of the water pipe and water fluid properties.

In general, permeability measurement systems suffer from several limitations when applied to tissue engineering. First of all, most of these systems use either water or oil as the fluid medium. Such systems require either a high induced pressure to force the fluid though or a long measurement time to allow the fluid to seep through. High induced pressure can deform porous scaffolds for soft tissue engineering and thus cause false readings. Long measurement time could cause complete saturation, which leave residual fluid in the sample and cause inaccurate results. Long measurement time is also a concern when water is used to measure biodegradable scaffolds, since the hydrolysis reaction can cause changes in the scaffold

itself. Furthermore, surface tension of water or oil could block small pores of the porous sample, causing lower permeability readings (58).

1.2.3. Permeability models

Permeability is a parameter involved in models of estimation of internal flow rate within a porous medium. Different models have been developed in which permeability appears as a key parameter. A few examples are given below.

Darcy flow transport model describes the flow of a fluid through a porous medium. Henry Darcy (108) revealed a linear proportionality between the flow velocity and the applied pressure difference. **Darcy model** is expressed by:

$$v = -\frac{k}{\mu}\frac{\partial P}{\partial x} \qquad \textbf{Eqn. 7}$$

where v, P, μ and k are the Darcy velocity (the mean fluid velocity over the cross section), fluid pressure, dynamic viscosity of the fluid and the permeability of the porous medium, respectively. The permeability k of the medium, which has dimension of L^2, depends only on the geometry of the medium. In three dimensions, Eqn. 7 can be generalized to

$$\vec{v} = \mu^{-1} K \nabla P \qquad \textbf{Eqn. 8}$$

where the permeability K is a general second-order tensor. The terms $\vec{v}$ and ∇P are Darcy velocity and pressure gradient vectors. For isotropic porous medium, the permeability is scalar and Eqn. 8 reduces to

$$\nabla P = -\frac{\mu}{k}\vec{v}. \qquad \textbf{Eqn. 9}$$

The Brinkman model is an extension to the traditional form of Darcy's law: in fact, Darcy model ignores fluid effects at boundaries. This assumption is not valid when the boundaries of the porous medium have to be accounted for. To overcome this limitation, the Brinkman model is usually employed

$$\nabla P = -\frac{\mu}{k}\vec{v} + \tilde{\mu}\nabla^2\vec{v} \qquad \textbf{Eqn. 10}$$

Eqn. 10 is referred in the literature as the **Brinkman model** and was first developed by Brinkman (109). The first viscous term on the right is the Darcy term while the second term on the right is analogous to the momentum diffusion term in the Navier–Stokes equation with $\tilde{\mu}$ being the effective dynamic viscosity of the medium.

Darcy model is limited by the fact that it neglects inertial component. Indeed, **Ergun-Forchheimer equation** is used (110) because it also describes an empirical relationship between the volumetric flow rate and the applied pressure differential across a porous medium.

It can be noticed that the pressure loss through the porous medium was due to a combination of frictional and inertial losses, which could be characterized using coefficients a and b as follows:

$$\frac{\Delta P}{L} = a\left(\frac{Q}{A}\right) + b\rho\left(\frac{Q}{A}\right)^2 \qquad \textbf{Eqn. 11}$$

where ΔP is the pressure differential, L is the thickness, A is the cross-sectional area of the porous sample and ρ is the fluid density. The difference between Darcy's law and Ergun equation is the addition of the second term in Eqn. 11 which accounts for the inertia effect of the flow. Without the second term, the equation goes back to

$$Q = A\frac{k(\Delta P)}{\mu L} \qquad \textbf{Eqn. 12}$$

Therefore, Darcy's flow Eqn. 12 is limited to a specific flow domain, whereas Ergun equation provides a complete description of the flow behaviours, including laminar, transitional and turbulent, through porous media.

1.2.4. Analytical and computational evaluation of permeability

The prediction of permeability *k* from theoretical work involves both models with simplified geometries, in order to obtain analytical solution of the microscopic flow pattern, and more sophisticated statistical methods. In literature, different correlations between permeability and parameters defining geometrical properties of scaffolds has been proposed, e.g. permeability as a function of porosity. In the last years, direct simulations of complex fluid flow phenomena is made possible by rapid development of computers and computational methods. For example, lattice Boltzmann methods are useful in simulating flows through irregular geometries. Within the numerical approach, correlations between permeability, porosity, effective porosity, specific surface area and tortuosity are often used to characterize mass transport in scaffolds.

For example, in Kozeny's equation, permeability is evaluated by the equation

$$k = \frac{c_1\varepsilon^3}{s^2} \qquad \textbf{Eqn. 13}$$

or, explicitly expressing tortuosity,

$$k = \frac{c_2\varepsilon^3}{Ts^2} \qquad \textbf{Eqn. 14}$$

where c_1 and c_2, that depend on the geometry of the cross-section of the capillary tube, are dimensionless constants, called Kozeny's constant. However, *T* and *s* are difficult to determine independently and *c* varies considerably depending on the sample because it is an empirical factor.

In his review, Karande (111) showed an empirical relation connecting permeability and porosity using different capillary models to represent the porous structure. In this model, permeability is evaluated as

$$k = \frac{\varepsilon^3}{T^2s^2} \qquad \textbf{Eqn. 15}$$

In their work, Botchwey and coworkers (112) represented the three dimensional (3D) architecture of scaffolds as interconnected spherical particles of radius r_g, encapsulated in a thin layer of fluid of radius, R_w

to evaluate shear stress inside a 3D micro-carrier scaffold. They assume that the flow through a scaffold with this pore structure obeys Darcy's law and, moreover, they suppose a homogeneous velocity profile, v_∞, at a large distance from the porous scaffold, that the normal vector of the scaffold front surface is parallel to the velocity at large distances from the disk, that the scaffold thickness is small and permeable to fluid flow, that the velocity, v, of the fluid in the scaffold is small compared with v_∞, and that the pressure gradient across the scaffold is approximated by the pressure difference per unit length along the scaffold, $\frac{\Delta P}{L}$. The permeability constant *k* is calculated as

$$k = \left[\left\{\frac{3 - (9/2)\gamma + (9/2)\gamma^5 - 3\gamma^6}{3 + 2\gamma^5}\right\}\frac{2{r_g}^2}{9\gamma^3}\right] \qquad \textbf{Eqn. 16}$$

where $\gamma = r_g/R_w$. γ can be expressed by the relation $\gamma = (1-\varepsilon)^{1/3}$. Based on physical characteristics of microcarrier scaffolds and measured scaffold motions in a rotating bioreactor, Eqn. 16 and Darcy's law Eqn. 7 can be used to calculate the volume flow rates and flow velocity of fluid perfusion through microcarrier scaffolds during dynamic culture. After that, shear stresses on the scaffold walls where calculated.

Anyway, this method does not give information about the local distribution of such stresses within complex 3D architecture of the real scaffold. Also in the computational fluid dynamic (CFD) study by Cioffi and coworkers (113), the value of permeability calculated by Darcy's law is used to evaluate shear stresses acting on the walls of the scaffold reconstructed by micro computed tomographty (μCT) (Fig. 24). In fact, they compared the average shear stress $\bar{\tau}$ obtained from the μCT based model with the value calculated using the analytical model of Wang and Tarbell, based on Brinkman's equation Eqn. 10 (114)

$$\bar{\tau} = \frac{\mu Q S^{-1}}{\sqrt{k}} \qquad \textbf{Eqn. 17}$$

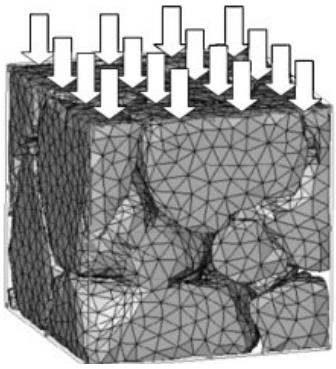


Fig. 24: CFD model of reconstructed scaffold (113).

where QS^{-1} is the flow average velocity at the scaffold inlet (ratio of flow rate and cross sectional scaffold area). They found that the analytical value of shear stress is higher than the average one obtained through CFD simulations.

In the work of Anderson (115), different scaffold design are created and CFD simulations are used to evaluate scaffold performance changing design parameters: in particular flow simulations are carried out to calculate permeability that is determined using Darcy's law

$$k = \frac{\dot{M}_{B1}\mu L}{A\rho P_{inlet}} \qquad \textbf{Eqn. 18}$$

where P_{inlet} is the applied inlet pressure. The idea of this study is that knowing how geometry affects flow conditions, the permeability of scaffolds can be a predictor of scaffold success for any design. In the study of Cantini et al. (116), the permeability of the scaffold is evaluated this way too. In this work, the authors use CFD techniques to optimize the microenvironment inside scaffolds for hematopoietic stem cell (HSC) culture in a perfusion bioreactor. They computationally design and study scaffolds with longitudinal microchannels to improve perfusion in a homogeneous porous scaffold (Fig. 25): their results show that these microchannels influence shear stresses and drag forces determining preferential paths for culture medium flow and forcing oxygen penetration into the scaffold bulk.

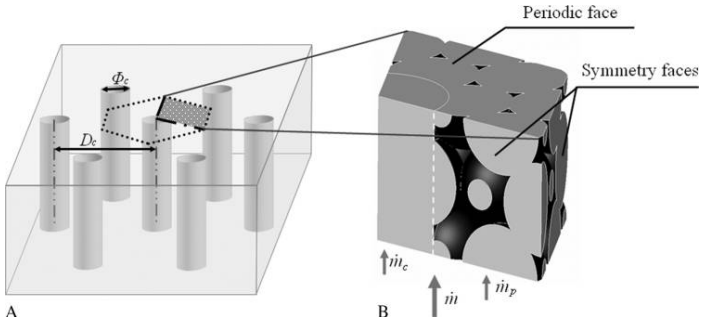


Fig. 25: Schematic representation of scaffold characterized by microchannels (116).

Liao and coworkers (117) used a finite difference technique to calculate the effective permeability tensor *K*.

In this study, the authors combine Darcy's Law and Navier - Stokes equation and, using the standard homogenization theory, they obtain the effective permeability of scaffolds designed with cubic or ellipsoidal interconnected pore architecture.

In their study, Ma and coworkers (118) developed a numerical model to examine the fluid flow condition (i.e. shear stress and nutrient distribution) within a scaffold placed inside a rotating wall perfused bioreactor (Fig. 26). In their model, laminar flow regime, Newtonian and incompressible fluid properties are considered, and the porous scaffold is assumed isotropic and homogenous, whose resistance is given by the Carmen–Kozeny theory

$$k = \frac{\varepsilon^3}{5s^2(1-\varepsilon)^2} \qquad \textbf{Eqn. 19}$$

where *s* can be defined as $s = \frac{\varepsilon}{R}$. They used this definition of permeability to solve the general porous flow equations.

In the study of Devarapalli (119), the nutrient distribution with consumption in high aspect ratio flow-through bioreactors containing porous structures is evaluated computationally. A modified Brinkman equation, that takes into account porous characteristics of the matrix, is solved on the porous regions and, taking in consideration the pore architecture of chitosan scaffold, the permeability was evaluated using an average pore radius r_p = 42,5 µm and n =120 pores/mm^2 as

$$k = \frac{\pi}{128} n_p (2r_p)^4 \qquad \textbf{Eqn. 20}$$

To study the influence of tissue remodelling, they simulate different scaffold decreasing pore sizes at constant number of pores per unit area: they confirm that the pressure drop is inversely proportional to $\frac{1}{k}$as predicted by the Brinkman equation.

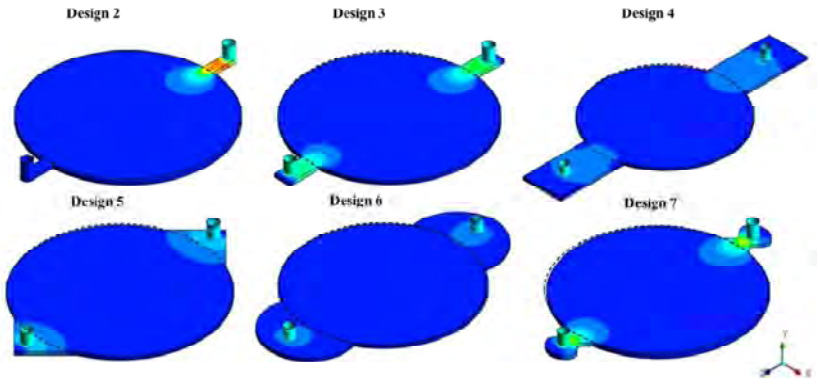


Fig. 26: Distribution of shear stress within high aspect ratio flow-through bioreactors (119).

Singh and coworkers (120) wanted to demonstrate the applicability of µCT on non-destructive quantification of several key features of scaffolds (Fig. 27). The authors evaluate and compare permeability obtained by experiment, CFD techniques and by Kozeny–Carman equation. In the first case, Darcian permeability is calculated using Dupuit–Forchheimer equation taking pressure value from hydrostatic head at the inlet and flow rate from a graduated cylinder at the outlet. In the second and the last case, µCT is used to obtain images from Ti foams and to reconstruct the model of the void zones of the scaffold. For the CFD simulation, the model was meshed and then simulation was performed to obtain the value of the pressure drop and flow rate used in the Darcy's law. Finally, the permeability is also calculated using the Kozeny–Carman equation as

$$k = \frac{\varepsilon^{\ 3}}{5s^2} \qquad \textbf{Eqn. 21}$$

where ε and s are measured from µCT data.

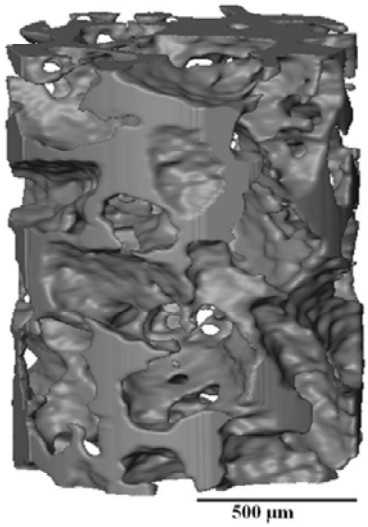


Fig. 27: Reconstructed scaffold from µCT (120).

Vossenberg and coworkers (121) used CFD to evaluate the flow field inside printed scaffolds (Fig. 28) and define Darcian permeability constant as a good predictor for the shear stresses in such scaffolds.

Fig. 28: Printed scaffolds characterized by cross disposition of fibres (121).

In this work, the Ergun-Forchheimer equation is used, explicitly expressing its constants as a function of darcian and non-darcian permeability

$$\frac{\Delta P}{L} = \frac{\mu}{k}\mathrm{v} + \frac{\rho}{k_2}\mathrm{v}^2 \qquad \textbf{Eqn. 22}$$

the empirical expressions of Darcian permeability constant for packed columns made of grains proposed by Ergun is used. In fact, Darcian permeability is defined as

$$k = \frac{\varepsilon^3 (2r_g)^2}{150(1-\varepsilon)} \qquad \textbf{Eqn. 23}$$

while non-darcian permeability is expressed as

$$k_2 = \frac{\varepsilon^3 2r_g}{1.75(1-\varepsilon)}$$ **Eqn. 24**

where ε is calculated as

$$\varepsilon = \left(1 - \frac{2\pi {r_g}^2 n_g}{wL}\right)$$ **Eqn. 25**

Printed scaffolds are characterized by fibres instead of grains so that to evaluate *k* the mean particle radius of the grains (r_g) has to be replaced by the radius of the scaffold fibres (r_f) as representation of the effective scale of the microstructure.

They found that reaching a critical value of permeability constant due to cell growth at the fibres may lead to a significant increase in shear stress. So the design of scaffold becomes important in order to maintain the same order of magnitude of shear stress over the cells.

1.2.5. Experimental measurement of permeability

Numerous experimental permeability measurement methods have been developed for determining the permeability of scaffolds for various applications. Methods such as physical/chemical gas absorption, mercury intrusion, epitermal neutron porosimetry and helium pycnometry can deliver information on density, pore size and porosity of a porous medium. However, they do not provide direct measurements on intrinsic permeability. Moreover, some of the measurements such as mercury intrusion porosimetry involve high pressures that could destroy the usually fragile structure of TE scaffolds (101). Due to these limitations, this paragraph aims at presenting different permeability measurement systems which use different types of fluid, devices and empirical laws describing the relationship between the volumetric flow rate and the induced differential pressure across a porous medium.

Ochoa et al.(122) developed a pressure-induced permeability test performed based on Darcy's law, used to assess the permeability of 45S5 Bioglass®-based scaffolds of 0.9 – 0.95 porosity for bone TE. The rig used to measure intrinsic permeability is shown schematically in Fig. 29.

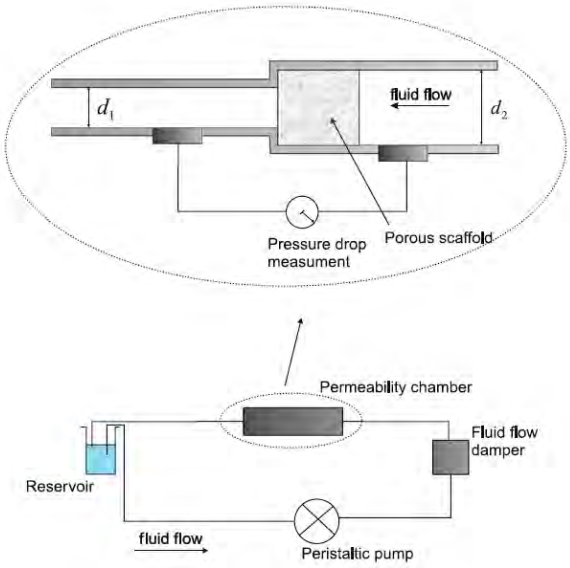


Fig. 29: Experimental rig used for permeability test to measure Darcy's permeability (122).

Deionized water (dH_2O) (μ = 10^{-3} Pa s), chosen as a fluid, is taken from an open reservoir to the air and is moved by a peristaltic pump. In order to obtain a continuous flow through the circuit, a fluid damper is used to limit the peristaltic pulse provided by the pump. The scaffold pressure drop is measured between two points, at the inlet and outlet of the redactor with the use of a pressure meter, in the permeability chamber that exhibits two different diameters, d_1 = 5.30 mm and d_2 = 8 mm, and the scaffold is located at the interface between both sections to avoid its relative movement due to the fluid stream. Different fluid flow regimes are applied and the corresponding pressure drop (Δp)-flow rate(Q) curves are obtained for both samples and the intrinsic permeabilities are estimated from those data. A similar system based on the use of $dH_2O$is used by Shimko et al. (123) to measure the permeability of twenty-one tantalum scaffolds for bone TE, fabricated as cylinders with porosities ranging from 0.66 to 0.88, in order to determine relationships for intrinsic permeability, tangent elastic modulus, yield behaviour, and microstructural geometry. After porosity measurements, each sample is carefully wrapped with Teflon-tape and gently inserted into a cylindrical, constant-pressure permeability testing chamber. Thanks to a large dH_2O reservoir suspended above the permeability chamber, a constant pressure head of 60 cm of dH_2O (~5.8 kPa) is applied to the upstream side of the chamber, while the downstream side of the chamber is exposed to atmospheric pressure. Prior to testing, 1000 mL of dH_2O is allowed to run through each sample to ensure full infiltration of fluid and removal of any trapped air bubbles within the structure. Immediately following this infiltration step, dH_2O is passed through the sample for 60 s and all dH_2O coming out the samples during the test is collected, and its total volume is recorded. This process is repeated six times for each samples, and intrinsic permeability is calculated using Darcy's law. The same method is used again by Shimko and Nauman (124) in order to evaluate the permeability of highly porous poly methyl methacrylate (PMMA) scaffolds, produced using a variant of the commonly used particulate leaching method, with controllable modulus and intrinsic permeability.

Further studies have been performed in the field of bone TE. To investigate the structure-function relationship for the permeability of coralline hydroxyapatite (HA), before, and trabecular bone (vertebral body, human proximal femur and bovine proximal tibia), after, Haddock et al. (125) and Nauman et al. (126) have used a constant flow rate permeameter (Fig. 30) to determine the intrinsic permeability of the specimens differently oriented.

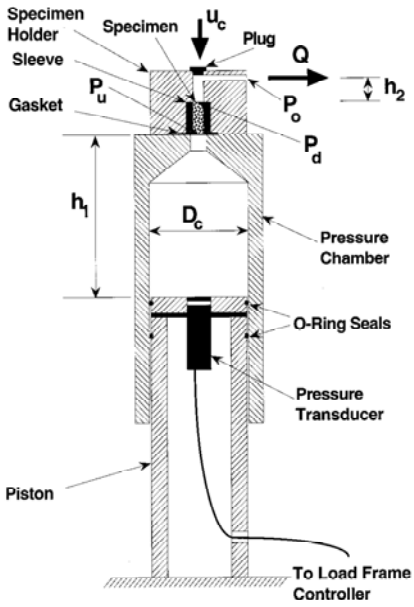


Fig. 30: Constant flow rate permeameter.

The movable cylindrical pressure chamber was pressed downward onto the fixed piston at a speed, uc, forcing fluid through the specimen at a know volumetric flow rate, Q. The pressure was measured at the piston face by a pressure transducer. The upstream, downstream and outlet pressures were P_u, P_d, and P_o, respectively (126).The apparatus consists of a specimen holder, a movable cylinder and a fixed piston. An air-tight seal is maintained by two O-rings and deionized and degassed water at room temperature is forced through the sample by using a servohydraulic load frame to move the cylinder downward with respect to the fixed piston at a constant crosshead velocity. Darcy's law is used to relate the area-averaged velocity, through the specimen in a given direction to the pressure drop in the same direction. Kholes et al. (127) developed a device which allows for the positioning of each cubic cancellous bovine bone in the range of porosities 80-90% within a laminar flow channel (Fig. 31).

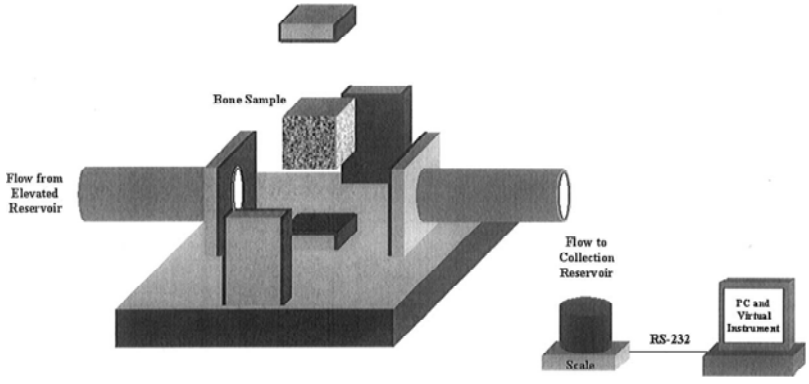


Fig. 31: An exploded-view schematic of the custom device used for direct perfusion of cubic, cancellous bone samples. The support pieces are pulled away from the bone sample for clarity. A constant pressure differential through each sample is created between the elevated and collection reservoirs. Additional support pieces securing the vertical and horizontal containment components are also absent for clarity. The shaded surfaces that are brought into contact with the bone sample surfaces represent a rubber gasket material that maintains uniform flow to the sample (127).

The intrinsic permeability of each samples is determined using an experimental setup which consist of an elevated reservoir that is used to drive water through the bone sample and into a collection reservoir. A

separate reservoir with an adjustable spigot spilled into the initial reservoir at a flow rate that maintains a constant, differential hydrostatic pressure through each sealed bone sample. The same gravity-driven method is used by Haugen et al. (128) and Sanz-Herrera et al. (129). The first one developed a device to measure the permeability of ceramic-based foams, using the height of a water pipe and fluid properties, and to link the permeability values to the degree of interconnectivity. Mercury intrusion porosimetry is used to measure interconnectivity and pore size distributions, whereas pore radius distribution is obtained using Washburn equation:

$$r_p = \frac{\left(\frac{-4\gamma cos\theta}{P}\right)}{2} \qquad \textbf{Eqn. 26}$$

where γ is the surface tension of mercury (480 dyne cm^{-1}), ϑ is the contact angle between mercury and TiO_2, which is taken to be 141°, and P is the pressure measured during the mercury intrusion process. The mean pore radius of the sample is calculated from the measured pore radius distribution, and its porosity is obtained from the total intruded volume.

Permeability is measured with a self-constructed permeability-meter in which water is used as flow media and the sample is located in fitting consisting of two half shells. Based on the percolation theory of Katz and Thompson (130) applied to laminar flow in porous media to develop a mercury porosimetry, the predicted ratio of the fluid permeability k to the electrical conductivity σ of a porous material was described by:

$$\frac{k}{\sigma} = \frac{cd_c^2}{\sigma_0} \qquad \textbf{Eqn. 27}$$

where σ_0 is the electrical conductivity of the pore saturating fluid, c is a calculated constant on the order of 0.01 that weakly depends on the assumptions made for the relationship between pore length and pore diameter and d_c is the threshold pore diameter as measured by mercury porosimetry. The electrical conductivities of the tested samples are unknown and have to be calculated from obtained mercury intrusion porosimetry data. Firstly, the characteristic length, L_{char}, must be determined and it is found from the threshold pressure, P_{thres}, using the Washburn equation (see Eqn. 26). The threshold pressure is the pressure at which the intrusion volume versus pressure curve is steepest, which is calculated by supplied product software. L_{char} is defined as the diameter of the pore that just completes the first continuous pathway through the material, if the pore space is sequentially built up starting with the largest pores working down. This pathway consists only of the pores with L greater than or equal to L_{char}. Katz and Thompson found experimentally that L_{char} closely corresponds to the inflection point on the cumulative intrusion curve.

The conductivity for the TiO_2 samples is calculated using the length at which conductance is maximum, L_{max}. The conductance is maximum when $(I - I_{thresh})(2r_p)^3$ is maximum, where I is the intrusion volume and I_{thresh} is the specific volume intruded at pores larger than L_{char}. I_{thresh} is calculated by interpolating the specific intrusion volume versus pore radius curve at L_{char}. The fractional volume of connected pore space involving pore widths of size L_{max} and larger, S_{Lmax}, can be calculated by interpolating the specific intrusion volume versus pores size curve to L_{max} and dividing by the total specific intrusion volume I_{tot}. Hence, the absolute permeability k can rewritten from Eqn. 27 and be calculated as follows:

$$k = \frac{1}{89} L_{max}^2 \cdot \frac{L_{max}}{L_{char}} \cdot I_{tot} \cdot Y_b \cdot S_{Lmax} \qquad \textbf{Eqn. 28}$$

where Y_b = sample weight/bulk volume.

The same approach is performed by Sanz-Herrera et al. (129) who uses a gravity-induced pressure test to measure the intrinsic permeability for three types of Sponceram® cell carriers. The developed method is independent of the fluid and, also in this case, may be related to the degree of interconnectivity of the scaffold microstructure. Therefore, an elevated reservoir to induce a controlled flow rate is set, while a permeability device is developed and manufactured to host the Sponceram® discs with two open holes to measure the pressure drop (Fig. 32).

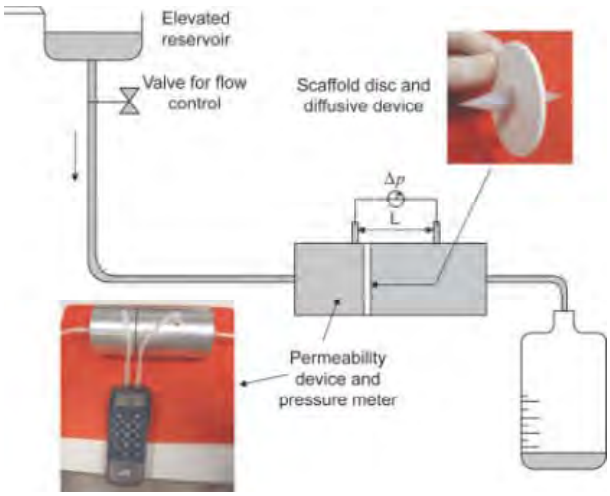


Fig. 32: Schematic representation of the permeability test (129).

Li et al. (59) performed a permeability study based on a method previously described by Grimm and Williams (62), in which linseed oil is used to flow through a bone sample by gravity-induced pressure. The permeability of the bone sample is determined based on the amount of linseed oil flowing through the sample within a certain period of time. Instead, in the study of Li et al. (59), a fluid under a known pressure is allowed to flow through the porous specimen and the flow rate is measured. Briefly, samples are prepared in the shape of either a rectangular bar or a cylinder, with a length of 20 mm and are connected with a fluid reservoir thanks to a piece of rubber tube. All specimens are mounted in a polystyrene (PS) tube, and the space between samples and the PS tube is sealed with parafilm. The pressure at the bottom surface of the specimen is zero, while the pressure at the top surface is generated by the water level between reservoir and specimen top surface. A maximum flow volume of 80 mL is used, resulting in a 5.7 mm drop in fluid level and a negligible pressure drop of 0.4% of the original pressure. The volumetric flow rate through the specimen is measured by determining the volume of water that flowed through the specimen into a 100 mL graduated cylinder in a span of time measured with a stop watch.

Based on the previous study, Wang et al. (131) measured the permeability of porous collagen scaffolds prepared by lyophilization and poly(ethyleneglycol terephthalate)-poly(butylenes terephthalate) (PEGT/PBT)-collagen hybrid scaffolds. A cylindrical scaffold (~16 x 2 mm) hydrated in demineralised water overnight is placed and fixed in the tube as indicated in Fig. 33.

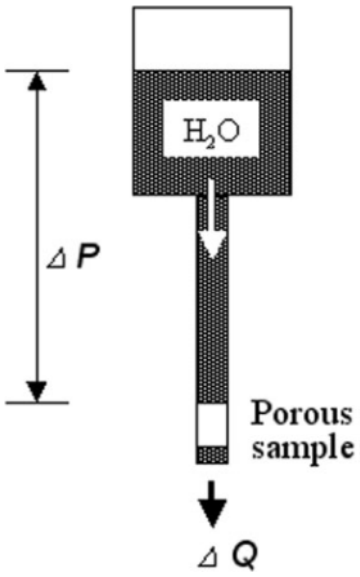


Fig. 33: The schematic diagram of how the flow permeability was measured (131).

Weight increase of water that passes through the scaffold is continuously electronically recorded and the flow permeability is calculated using Darcy's law.

Sell et al. (132) developed a flowmeter that can accurately measure the hydraulic permeability of electrospun fibrinogen scaffolds with three different fibre diameters and pore sizes. The device and method is a modified version of the technique realized by Carr et al. (133), (134) and by Wang et al. (131) previously described. This required the design and construction of a flowmeter and specimen mount to measure the amount of fluid passed through a fixed electrospun scaffold area over time (Fig. 34).

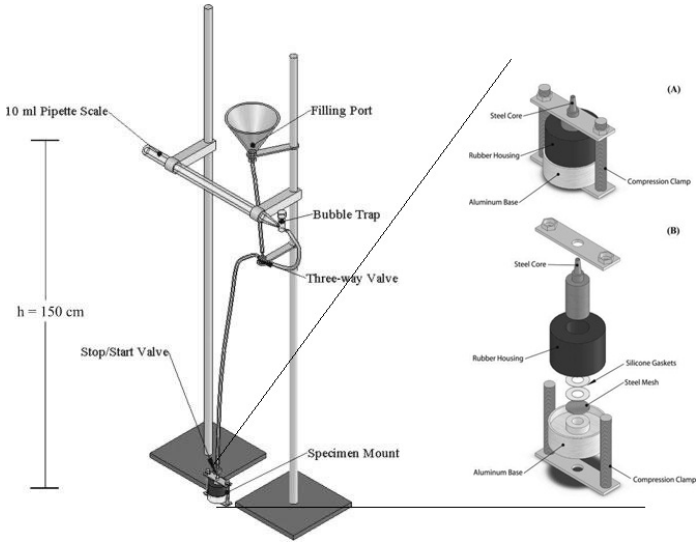


Fig. 34: Diagram of the simple flowmeter used in this study. Modified from the flowmeter of Dr. Carr, designed for testing fibrin gel permeability(left). Flowmeter specimen mount in collapsed (A) and exploded views (B). Electrospun scaffolds are placed between the two silicone gaskets for testing in order to create a tight seal around the desired area of fluid flow. Steel mesh is placed beneath the scaffold and gaskets to reduce scaffold distension(right) (132).

As electrospun scaffolds have high porosities, allowing water to migrate outside of the intended area of fluid flow and into surrounding areas of the scaffold, it is necessary to create a bound area of fluid flow. The

bulk of the flowmeter presents a graduated 10 mL pipette placed horizontally as the measurement scale. To create a larger pressure head and push fluid through the tightly packed electrospun scaffolds faster, the height *h* (distance between horizontal pipette and scaffold) is increased from 20 to 150 cm.

A novel specimen-mounting apparatus was fabricated, in order to support an electrospun scaffold. To ensure that all fluid travels through a set area of the electrospun scaffold and produces accurate permeability values, the mounting apparatus is designed to work by compressing the outer edge of the scaffold between two silicone gaskets. The compression is intended to form a tight seal and force all fluid flow through the open centre of the specimen mounting apparatus. A large pore metal screen is placed on the underside of the scaffold to prevent excessive distension of the test scaffold, which would again alter the cross-sectional area of fluid flow and effect permeability measurement.

Concerning the permeability measurement, scaffolds both dry and hydrated are individually placed in the flowmeter specimen mount. The flowmeter tubing and the pipette are filled with PBS and permeability testing is begun at room temperature (~25°C). Fluid flow is measured using the incremental marks on the 10 ± 0.05 mL pipette and is recorded every minute for the first 10 min of testing. After the initial 10 min, recordings are made at 15 min and 20 min, in addition the total time for 13 mL (maximum volume of pipette) to pass through the scaffold is recorded. These time values are then used in the following Darcy's equation to determine scaffold permeability:

$$k = \frac{Q\mu L}{AtP} \qquad \textbf{Eqn. 29}$$

where μ is 0.89 cP for water at 25°C and *P* is the applied pressure head. The applied pressure head *P* is determined by using the following equation:

$$P = \rho g h \qquad \textbf{Eqn. 30}$$

where ρ, the density of water, is 1000 kg/m^3 at 25°C, *g* is the gravitational force (9.8 m/s^2), and *h* is the total height of the system in meters (1.5 m). This pressure must then be converted from pascal (Pa) to atmospheres (atm) for use in Darcy's equation.

Wang et al. (135) compared different methods for determining permeability coefficients in a microporous tubular scaffold produced from poly(caprolactone) (PCL). The coefficients are derived from measurements of the amount of fluid (air and water) that flowed through the material in a specified time for a given pressure differential. The permeability coefficient for air of the PCL scaffold material is obtained by passing air through a microporous PCL sample in the form of a 1 cm diameter disc, approximately 1mm thick, at rates ranging from 100 to 800 cm^3/min and measuring the pressure drop across the disc as shown in Fig. 35.

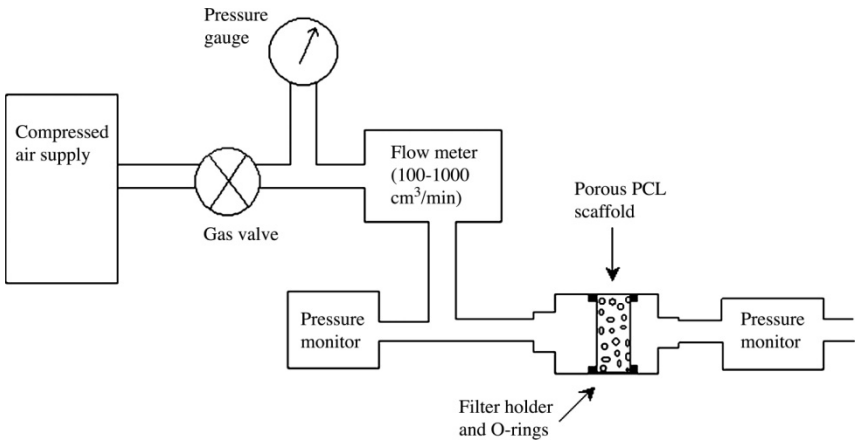


Fig. 35: Measuring the pressure differential across a disc of microporous polycaprolactone (PCL) scaffold material produced by the controlled flow of gas through the disc (135).

Samples are mounted onto a filter holder using two O-rings and connected to a compressed air supply. The airflow rate is controlled using a mass flow controller, and the pressure drop is measured using a pressure controller. In order to determine the Darcy permeability coefficients for water and in order to overcome the impermeability to water of PCL at moderate pressures of 12,000 Pa, the microporous tubes are immersed in a 20% v/v ethanol/water mixture for 30 min, followed by two washes in distilled water before testing. The permeability coefficient of water through the microporous PCL scaffold is determined from Darcy's law using measurements of the weight of water that permeates through the tube wall in a given time under a given pressure. Tubes are mounted in a Bose-Enduratec BioDynamic chamber (Bose, Eden Prairie, MN) (Fig. 36).

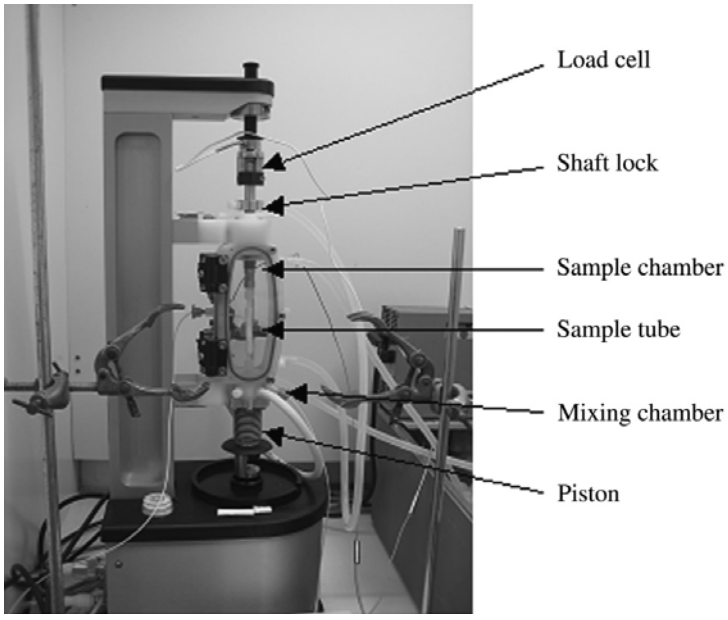


Fig. 36: Bose-Enduratec BioDynamic chamber (135).

Fluid is pumped from a reservoir to a mixing chamber before passing through the tube at selected flow rates in the range 0– 1500 mL/min. All measurements are performed at 37°C. The sample chamber is filled with degassed distilled water to maintain a constant pressure head (Fig. 37).

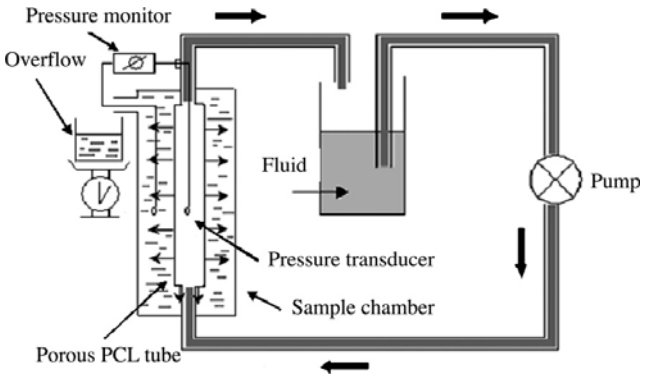


Fig. 37: Passage of fluid through the wall of a microporous PCL tubular scaffold in a closed loop pumped system determined by either weighing the permeant or measuring the change in concentration of a molecular probe (135).

Two pressure transducers, one positioned inside the tube mid-way along the tube length and the second located external to the PCL tube (Fig. 37), are used to continuously record the pressure drop across the tube wall. Fluid that passes through the wall of the PCL tube is collected as overflow from the sample chamber over a specific time interval to determine the permeation flow rate (*Q*) under various pressures. Finally, they used the same method to determine the Darcy permeability coefficient for molecular probes, such as ions, glucose, or proteins used to determine the rate of fluid flow through a permeable matrix. This approach is advantageous in those measurements of permeability of small molecules and could provide useful data on nutrient and metabolite transport through a scaffold. In addition, the rate of movement and distribution of polypeptide growth factors such as vascular endothelial growth factor and chemoattractants through the scaffold are relevant to processes of cell development and tissue ingrowth. A solution containing the selected molecular probe is circulated through the sample tube mounted in the Bose - Enduratec BioDynamic chamber. The concentration of each selected molecular species is determined in the fluid that overflowed from the sample chamber at intervals ranging from 10 to 60 s, depending on the pressure differential across the tube wall. The cumulative amount of the molecular probe that passes through the tube wall over time is subsequently calculated from the volume of the sample chamber and the overflow. All measurements are performed at 37°C under constant flow rate conditions through the tube (300mL/min) and at pressure differentials ranging from 10 to 80mm Hg (1.3–10.6 kPa). A small magnetic stirrer is placed in the sample chamber to avoid any concentration gradients that may otherwise have developed.

Phosphate-buffered saline (PBS) contains charged species (sodium and potassium cations, hydrogen phosphate, dihydrogen phosphate, and chloride anions) that can be used as molecular probes to determine the permeability of microporous materials. PBS is circulated through a microporous PCL tube mounted in the BioDynamic chamber, and the movement of charged species across the tube wall into the surrounding deionized water medium is detected as a change in resistivity or conductivity. Measurements are made between a 50mm platinum wire electrode located within the sample tube and a second electrode positioned within the sample chamber. Changes in conductivity are measured using a Precision impedance analyzer, and the data are recorded using Labview 8.0 (National Instruments, Austin, TX).

Always based on the Darcy's law, it is possible to measure the permeability of controlled pore structures realized by 3-dimensional printing and injection molding. Lee et al. (136) fabricated, by computer-aided design (CAD), three different 3D porous poly(propylene fumarate) (PPF) scaffolds with controlled pore size architecture (300, 600 and 900 μm), and they determined the permeability using the falling head

conductivity test. Scaffolds are mounted to a Teflon holder, which is vertically connected to a burette held on a steel stand. Before the test, they determine V_{max} (maximum volume of the burette) and V_i (burette volume at a certain time t_i), and measure h_{max} (height of the burette's maximum volume), h_{drip} (height of the drip point), and L_b (distance from V_{max} to V_i in the burette). From these values, Δh_o (initial head loss at zero time) and Δh_i (differential head loss at a certain time) are calculated by:

$$\Delta h_o = h_{max} - h_{drip} \quad \textbf{Eqn. 31}$$

$$\Delta h_i = h_{max} - \left[(V_{max} - V_i)\, x\, \left(\frac{L_b}{V_{max}}\right) + h_{drip}\right] \quad \textbf{Eqn. 32}$$

Then they fill the burette with water until V_{max}, as the end of the scaffold holder is closed, and measure the time when the water run through the scaffold until V_i. The hydraulic conductivity (H) is calculated by:

$$H = \left(\frac{aL}{At_i}\right) ln\left(\frac{\Delta h_o}{\Delta h_i}\right) \quad \textbf{Eqn. 33}$$

where a is the area of burette. Finally, the intrinsic permeability is determined by:

$$k = \frac{H\mu}{\rho g} \quad \textbf{Eqn. 34}$$

where μ (the viscosity of water at 20°C) =1.00 cP, ρ (the density of water) =1.00 g/cm^3, and g is the gravitational acceleration.

Other methods have been proposed for measuring the permeability of scaffolds fabricated with rapid prototyping techniques. Kemppainen (137) examined how the physical property of permeability, as described by Darcy's law, affects chondrocytes or bone marrow stromal cells seeded onto PCL scaffolds and cultured *in vitro*, with the hypothesis that this parameter can be used to more accurately predict the effects of scaffold architecture on chondrogenesis. In order to pursue this hypothesis, they use stable solid freeform (SFF) techniques to create 3D-designed poli(ε-caprolactone) scaffolds with significantly different permeability values ("low", "mid" and "high" permeable design) created through changing the diameter of the interconnection between pores. For the empirical permeability measurement a custom chamber was designed (Fig. 38) to exert a constant hydraulic pressure on a scaffold.

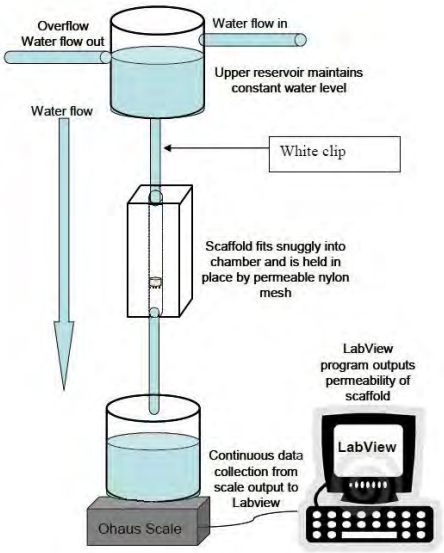


Fig. 38: Schematic representation of the custom permeability (137).

Water flow through the chamber and into a secondary container placed atop a scale connected to a PC is continually recorded. Within LabView, an equation derived from Bernoulli's equation (with a frictional loss correlation term) and Darcy's law, is used to compute permeability:

$$k = \frac{L}{A \cdot \dot{M_{B2}}} \cdot \frac{2\pi^2 R^4}{\left(\frac{\dot{M_{B1}}}{\dot{M_{B2}}}\right)^2 - 1} \qquad \textbf{Eqn. 35}$$

Where $\dot{M_{B1}}$= mass flow rate without scaffold (g/s), $\dot{M_{B2}}$ = mass flow rate with scaffold (g/s), R_w = radius of the water outlet (m). LabView records three reading per second, giving a continuos output of permeability. Permeability data is then plotted to validate that the permeability level was constant throughout the experiment and the data are averaged to obtain experimental scaffold permeability. Subsequently, based on this system, also Jeong and Hollister (138) measured the permeability of seven different porous Poly(1,8 Octanediol-*co*-Citrate) (POC) scaffolds.

Based on an alternative approach, Swider et al. (139) developed a high-resolution MRI methodology for characterizing the permeability and fluid velocity within an hydroxyapatite block cylindrically tooled of interconnected porosity.

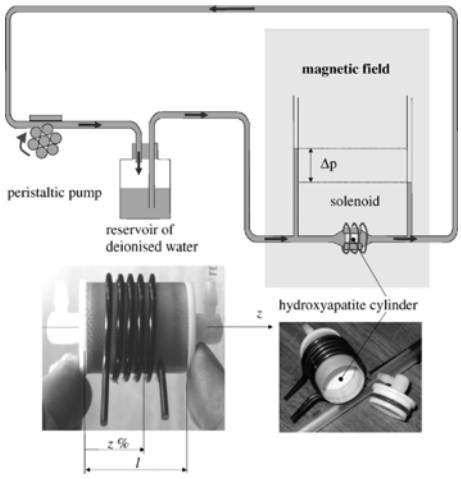


Fig. 39: Schematic of the fluid loop (139).

The flow circuit (Fig. 39) consists of a peristaltic pump and pressure reservoir to dampen pulsations to create a constant fluid flow, and two capillaries to measure the pressure gradient derived from the difference between upstream and downstream pressure. The sample is sealed in a specially constructed MRI probe consisting of a flow chamber surrounded by a solenoid RF coil and positioned in the MRI scanner.

The following methods describe studies based on equations that differ from the Darcy's law, for example, Ergun-Forchheimer equation.

Moreira et at. (140) tested the permeability of different ceramic foams made of SiC-Al_2O_3 utilizing air and water as flowing fluids. Ergun-Forchheimer equation is described as:

$$\frac{\Delta P}{L} = \frac{\mu}{k} U + \frac{\rho}{k_2} U^2 \qquad \textbf{Eqn. 36}$$

The pressure drop *ΔP* is given by

$$\Delta P = P_i - P_o \qquad \textbf{Eqn. 37}$$

in the case of incompressible fluid and by

$$\Delta P = \frac{P_i^2 - P_o^2}{2P_o} \qquad \textbf{Eqn. 38}$$

for compressible fluid (ideal gases), where P_i and P_o are the absolute pressures at the bed entrance and exit, respectively. P_i is the reference pressure for the density and viscosity of the flowing fluid.

The parameters *k* and k_2 are assumed to incorporate the structural properties of the medium, and to be a function of the bed characteristics only.

An Ergun-type correlation is fitted to the data and the proposed equations describe *k* and k_2 for packed columns made of spheres, cylinders, tablets, nodules, round sand and crushed materials as follows:

$$k = \frac{\varepsilon^3 d_p^2}{150\,(1-\varepsilon)^2} \qquad \textbf{Eqn. 39}$$

$$k_2 = \frac{\varepsilon^3 d_p}{1.75\,(1-\varepsilon)} \qquad \textbf{Eqn. 40}$$

The general view of the equipment used for permeability measurements of the ceramic foams is illustrated in Fig. 40 for both the fluids used in the experiment (water and air) at ambient temperature.

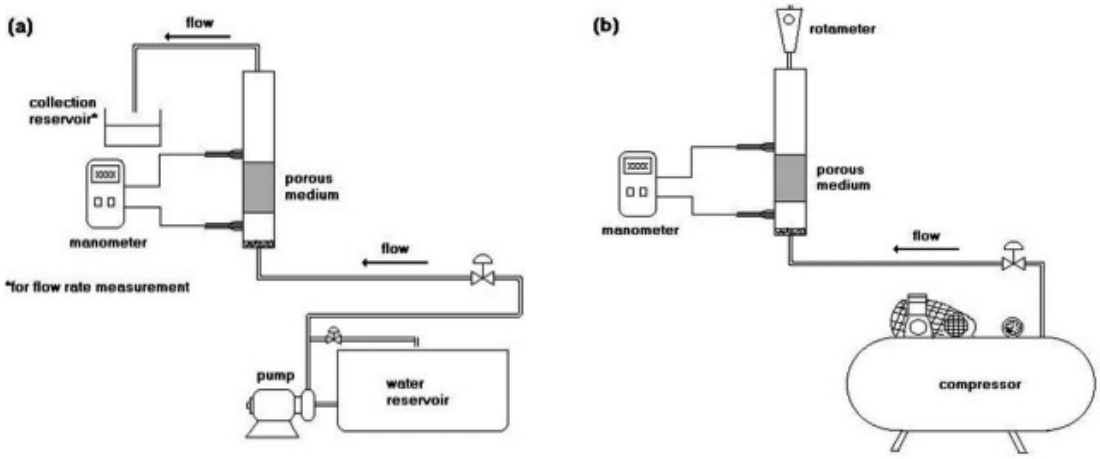
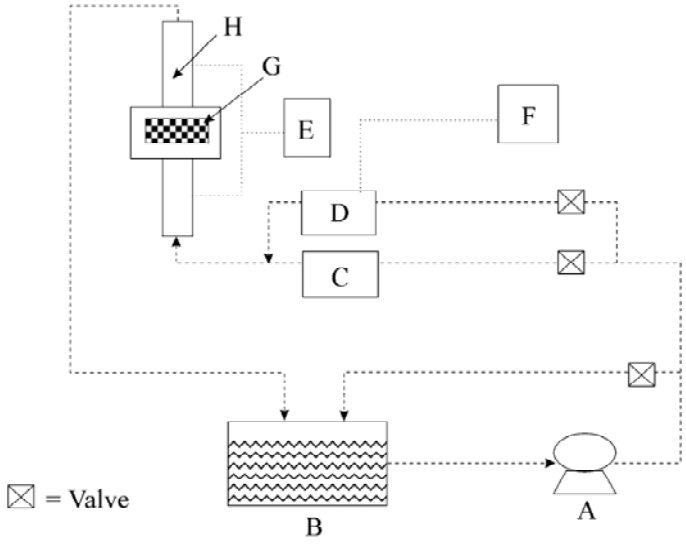


Fig. 40: General view of the equipments for the permeability measurements of (a) water and (b) air (140).

For water permeability measurements, the mass flow rate is obtained by weighing water that flowed through the sample in a given period of time. For air permeability, the mass flow rate is calculated from the volumetric flow, measured by a rotameter, and corrected for the temperature and pressure. In both cases, the pressure drop is measured utilizing a micro - manometer. The tested samples are 8, 20 and 45 pores per inch (ppi) ceramic foams disks with diameter of 7.5 cm and thicknesses of 3.0, 2.9 and 1.4 cm, respectively. The experiments are made in triplicates and the pressure drop is plotted as function of the fluid superficial velocity, *U*. The same experiment was performed by Innocentini and Faleiros (141) to test the permeability of synthetic ceramic (HA) and metallic (Ti) scaffolds, produced by gelcasting, that mimic trabecular bone. Only air at room temperature is used as fluid in the experiment. Previously, always based on Ergun-Forchheimer equations, Innocentini et al. (142) calculated the permeability of ceramic foams with 30 to 75 ppi and compared the values with those obtained experimentally under water flow. Experimental pressure drop (*ΔP*) across each sample was measured using the apparatus schematized in Fig. 41.



A - Centrifugal pump, 1 Hp

B - Water reservoir, 150 l

C - Electronic flow meter, Q = 0 to 800 l/h

D - Pitot tube flow meter, Q < 5000 l/h

E - U-type manometer, ΔP = 0 to 500 mmH

F - U-type manometer, ΔP = 0 to 500 mmHg

G - Sample disk (ϕ = 7.5 cm, L = 2.5 cm)

H - Sample holder (flow diameter = 5.91 cm)

Fig. 41: Apparatus for experimental determination of ceramic foam permeability to water flow (142).

Chor and Li (101) developed a permeability measurement system for TE scaffolds able to achieve rapid measurement operation without introducing any hydrolysis effect to the porous microstructure using a nonlinear least-squares procedure for parameter estimation. The proposed horizontal measurement system (Fig. 42) used dry air as the fluid medium.

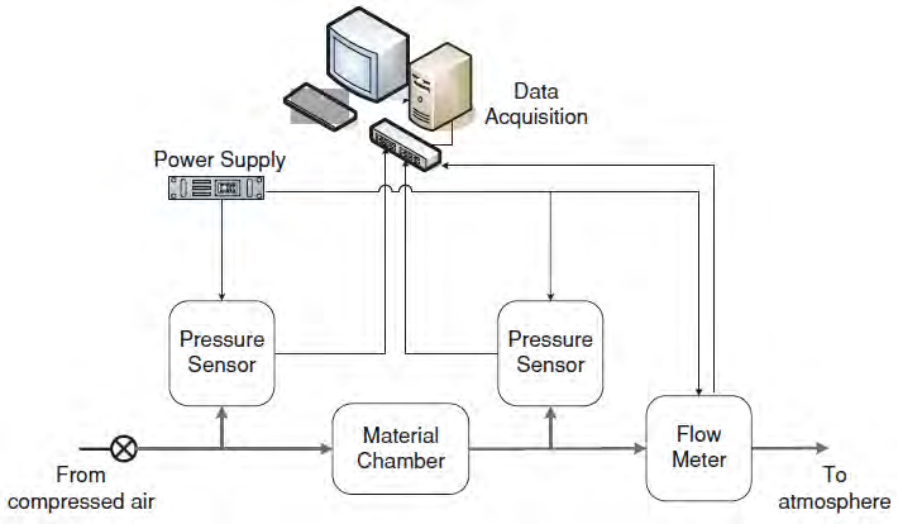


Fig. 42: A schematic of the permeability measurement system for TE scaffolds (101).

The scaffold sample is prepared in disc shape and is held inside a cylindrical material chamber, across which the pressure differentials is combined with flow rate measurements to determine the effective porosity, from which the intrinsic permeability is derived.

Using physical parameters, in this work, Ergun-Forchheimer equation Eqn. 11 is expanded into

$$\frac{\Delta P}{L} = 150\frac{(1-\varepsilon_e)^2}{{\varepsilon_e}^3}\frac{\mu \mathrm{v}}{{d_p}^2} + 1.75\frac{\rho(1-\varepsilon_e)}{{\varepsilon_e}^3}\frac{\mathrm{v}^2}{d_p} \qquad \textbf{Eqn. 41}$$

Eqn. 41 can be rewritten into

$$\Delta P = 150\frac{(1-\varepsilon_e)^2}{{\varepsilon_e}^3}\frac{\mu \mathrm{v} L}{{d_p}^2} + 1.75\frac{\rho(1-\varepsilon_e)}{{\varepsilon_e}^3}\frac{\mathrm{v}^2 L}{d_P} \qquad \textbf{Eqn. 42}$$

while Eqn. 12 can be rewritten into

$$\Delta P = \frac{\mu L}{k}\frac{Q}{A} = \frac{\mu L}{k}\mathrm{v} \qquad \textbf{Eqn. 43}$$

Since Darcy's law only describes the linear portion of the pressure differential as a function of the flow velocity, the linear terms of Eqn. 42 and Eqn. 43 can be equated. This leads to the following relationship between the intrinsic permeability in Darcy's flow region and the effective porosity of the porous medium:

$$k = \frac{d_p^2 {\varepsilon_e}^3}{150\ x\ (1-\varepsilon_e)^2} \qquad \textbf{Eqn. 44}$$

Eqn. 44 suggests that the intrinsic permeability can be obtained once the effective porosity and pore diameter are known. Since the effective porosity ε_e can be estimated using Ergun equation, the intrinsic permeability can then be obtained without observing the low flow rate constraint.

Instead, if the flow velocity is limited such that a small interstitial Reynolds number is maintained, the intrinsic permeability can be directly obtained from the measurement data. For a 10% linearity error, the Reynolds number has to be smaller than 8.6. With the estimated effective porosity, the interstitial Reynolds number can be determined using the following equation for each pair of the data points for pressure differential and flow rate:

$$Re_i^* = \frac{\rho \varphi D_p U}{\mu(1-\varepsilon_e)} \qquad \textbf{Eqn. 45}$$

If the interstitial Reynolds number is smaller than 8.6, the data point is accepted. Otherwise, the data point is removed from the recorded data list. The remaining data points were used to best fit a straight line. The slope of *Q* versus *ΔP* is used to calculate the intrinsic permeability *k* as:

$$k = \frac{c\mu L}{A} \qquad \textbf{Eqn. 46}$$

where *c* is the slope of the fitted line.

Further studies have been performed in order to compare the permeability values obtained both with experimental and analytical methods. O'Brien et al. (63) characterized the permeability/fluid mobility of collagen-GAG (CG) scaffolds as a function of pore size and compressive strain, to investigate potential factors that influence construct permeability, to relate scaffold permeability data to previously observed changes in cellular adhesion within these scaffolds, and to develop a cellular solids model capable of

quantitatively describing the permeability characteristics of a variety of scaffold microstructures under different loading conditions. They hypothesize that the permeability of these scaffolds is inversely proportional to the specific surface area due to the frictional effects of fluid flow by the struts that define the scaffold microstructure. In this study, they aim at testing this hypothesis by (i) characterizing the permeability and fluid mobility of the same series of CG scaffolds, (ii) investigating the effect of pore size and compressive strain on scaffold permeability, and (iii) developing a cellular solids model to mathematically describe construct permeability in terms of significant structural factors (e.g. mean pore size, applied strain, relative density). The permeability of four CG scaffolds with four distinct mean pore sizes (151, 121, 110, 96 µm) is tested as a function of mean pore size and compressive strain using both (i) an experimental technique and (ii) mathematical modelling technique using a cellular solids modelling technique used previously to successfully describe the variation of CG scaffold specific surface area with mean pore size. For the experimental permeability a device (Fig. 43) was constructed to measure the permeability of the scaffold variants under different levels of applied compressive strain.

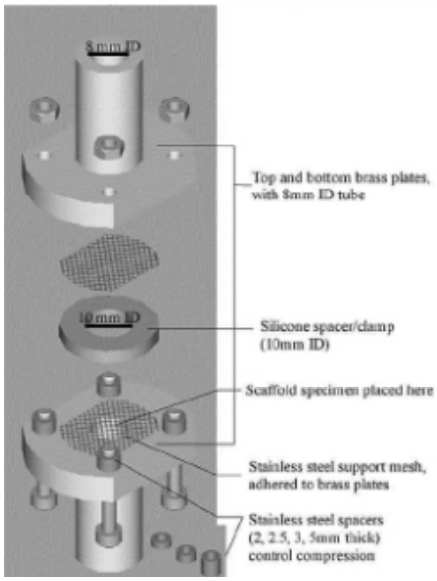


Fig. 43: Device utilized in this study to measure the permeability (fluid mobility) of collagen-GAG scaffolds. Stainless steel spacers of varying thickness regulated the degree of applied compressive strain while preventing fluid loss from the scaffold edges. The fours spacers of 5, 3, 2.5 and 2.1 mm give resultant strains of 0%, 14%, 29% and 40% (63).

The device is constructed from two (top and bottom) brass plates, each with an attached 8 mm inner diameter tube. A stainless steel mesh is secured over the tube openings in both brass plates. The mesh, a medical-grade stainless steel is adhered to the brass plates with a high-grade cyanoacrylate glue. The mesh, that do not inhibit or disrupt fluid flow through the testing rig, serve a two-fold purpose: to physically support the scaffold above the tube opening in the bottom plate, and to apply compressive strain to the scaffold via the height-adjustable top plate. Stainless steel spacers of varying thickness are placed between the two stainless steel meshes when the testing rig is assembled, regulating the degree of applied compressive strain. To secure the scaffold in place, the scaffold edges are clamped to the bottom stainless steel mesh using a silicone spacer which also serve as a leak-proof seal for the rig, preventing pressure loss. The inner diameter of the silicone spacer is slightly larger than that of the brass plate. Cylindrical scaffold disks, 13 mm in diameter and 3.5 mm thick, are cut from each scaffold variant using a dermal punch and then submerged in saline solution for 24 hours prior to testing in order to completely hydrate the scaffold. The permeability (k) and fluid mobility (S) of each of the four scaffolds under each level of applied

compressive strain was calculated using Eqn. 18, a modified version of Darcy's equation where Q is the measured flow rate, μ is the saline solution viscosity, r the radius of the scaffold sample (4 mm), L the thickness of the sample, and h the height of the saline solution column (1.2 m).

$$k = \frac{324.8(Q \cdot L \cdot \mu)}{r^2 h} \quad \textbf{Eqn. 47}$$

$$S = \frac{k}{\mu} = \frac{324.8(Q \cdot L)}{r^2 h} \quad \textbf{Eqn. 48}$$

From the mathematical permeability, a low-density, open-cell foam cellular solids model utilizing a tetrakaidecahedral unit is used to model the permeability of CG scaffolds with variable mean pore size under variable applied compressive strain. They have previously shown that cellular solids modelling techniques using a tetrakaidecahedral unit cell can accurately represent and predict salient microstructural features of CG scaffolds. This modelling is possible because the pore structure of a variety of low-density, open-cell foams has been observed to have three consistent features: an average of 14 faces per unit cell, 5.1 edges per face, and vertices that are nearly tetrahedral. These morphological features are observed as a result of a minimization of the total surface area of each unit cell. The tetrakaidecahedron is a polyhedron that packs to fill space, approximates the structural features of many experimentally characterized low-density, open-cell foams, nearly satisfies the minimum surface energy condition, and is often used for modelling such foams. In addition, the value of the dimensionless measure of total edge length per (unit volume)$^{1/3}$ for the tetrakaidecahedral unit cell is nearly identical to that observed for many random cellular structures, suggesting that the tetrakaidecahedral unit cell gives a good representation of the total edge length and can be used to model the specific surface area of random cellular structures such as the porous, collagen-GAG scaffold.

A quantitative, cellular solids model describing the permeability (k) of, or fluid mobility (S) through, CG scaffolds in terms of scaffold mean pore size (r_p), individual strut length (l), percent compression (η), a dimensionless system constant (A'), and scaffold relative density (ρ^*/ρ_s) has been developed from a series of known cellular solids relationships. The permeability (k) of many open-cell foams has previously been reported in terms of mean pore radius (r_p) and relative density (ρ^*/ρ_s, 1 – porosity):

$$k = A' \cdot (2r_p)^2 \cdot \left(1 - \frac{p^*}{p_s}\right)^{\frac{3}{2}} \quad \textbf{Eqn. 49}$$

This model can be modified to describe scaffold fluid mobility (K) using a single, system constant (A'', $A'' = A'/\mu$) with dimensions $(\text{Pa}\cdot\text{s})^{-1}$:

$$S = \frac{A'}{\mu} \cdot (2r_p)^2 \cdot \left(1 - \frac{p^*}{p_s}\right)^{\frac{3}{2}} = A'' \cdot (2r_p)^2 \cdot \left(1 - \frac{p^*}{p_s}\right)^{\frac{3}{2}} \quad \textbf{Eqn. 50}$$

The mean pore size of the compressed scaffold ($d_{p\text{-}compress}$) under a given applied compressive strain (η_{app}) can be defined in terms of the initial edge length of the unit cell used to describe the scaffold microstructure (l). A relationship between applied compressive strain and compressed mean pore size, modified from that proposed by Gent and Rusch (143) for this CG scaffold system with an observed Poisson's ratio of 0, is presented here:

$$d_{p-compress} \propto l \cdot (1 - \eta_{app}) \quad \textbf{Eqn. 51}$$

Additionally, for the tetrakaidecahedral unit cell, scaffold mean pore size (r_p) can be related to the average edge length of an individual strut (l) within the unit cell:

$$r_p = \frac{2.875 \cdot l}{2} \quad \textbf{Eqn. 52}$$

Combining these relationships, a single model describing scaffold permeability (k) or fluid mobility (S) in terms of a system constant (A' *or* A''), scaffold mean pore size (r_p), applied compressive strain (η_{app}), and relative density (ρ^*/ρ_s) that is valid for any low-density, open-cell foam is proposed. These models are identical with the exception of the single, system constant ($A'' = A'/\mu$):

$$k = A' \cdot \left(\frac{2r_p}{2.875}\right)^2 \cdot (1-\eta)^2 \cdot \left(1 - \frac{p^*}{p_s}\right)^{\frac{3}{2}} \quad \textbf{Eqn. 53}$$

$$S = A'' \cdot \left(\frac{2r_p}{2.875}\right)^2 \cdot (1-\eta)^2 \cdot \left(1 - \frac{p^*}{p_s}\right)^{\frac{3}{2}} \quad \textbf{Eqn. 54}$$

Al-Munajjed et al. (144) developed a similar study based on the previous work. The target of this investigation is to analyze the influence of pore size on the experimental and analytical permeability for three different types of scaffolds (Table 2) for which the basic component, mixed with NaCl crystals, is a spongious matrix consisting of 70% esterified hyaluronal and 30% collagen.

Table 2: Survey of the used scaffold structure geometries (144).

	Scaffold A (µm)	Scaffold B (µm)	Scaffold C (µm)
Salt crystals	250-355	355-450	450-600
Mean pore size	302.5	402.5	525

Concerning the experimental permeability, an experimental device (Fig. 44) was realized.

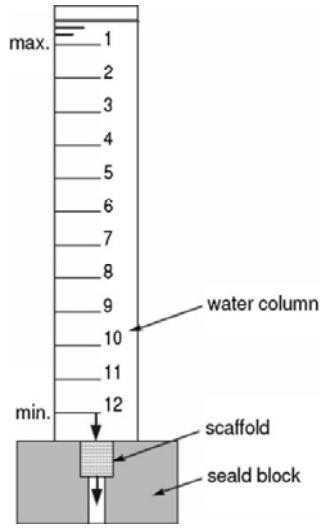


Fig. 44: Schematic view of the set-up for the experimental permeability tests. The medium flows from the Plexiglas tube through the scaffold in the polyvinyl chloride holder to a beaker (144).

The device is constructed from a 260-mm long upper Plexiglas tube and a bottom scaffold holder. An aliquot of 0.9% NaCl is chosen as the medium for permeability in the system. The tube is fixed via a press fit connection to the scaffold holder, which is made out of polyvinyl chloride with an inner tube for the scaffold fixation. The inner tube has a diameter of ~6.5 mm to ensure a slight press fit of the scaffolds (diameter 6.8 mm). Time periods between the marks on the Plexiglas tube are measured and the permeability is calculated according to Darcy's equation. For mathematical permeability a tetrakaidecahedral unit according to O'Brien et al. (63) and Gibson and Ashby (145) is used to calculate the permeability. O'Brien et al. (63) have previously shown that cellular solids modelling techniques using a tetrakaidecahedral unit cell can accurately represent and predict salient micro structural features of collagen scaffolds. In this investigation Eqn. 53 is used, but no compression of the scaffolds is performed ($\eta = 0$), therefore $(1 - \eta)^2$ was set as 1 obtaining:

$$k = A' \cdot \left(\frac{2r_p}{2.875}\right)^2 \cdot 1 \cdot \left(1 - \frac{p^*}{p_s}\right)^{\frac{3}{2}} \qquad \textbf{Eqn. 55}$$

1.3. Scaffold computational characterization

1.3.1. Porosity and Computational Flow Analysis for Collagen-membrane and Cross-Linked Gelatin Scaffolds using a high contrast MicroCT with submicron resolution

This study was focused on developing a rapid 3D imaging technique to characterize porosity and fluid dynamics in collagen membrane and cross-linked gelatin composite scaffolds for tissue regeneration.

Fluid dynamic transport properties, namely porosity and permeability, are a main topic for bioresorbable scaffolds, designed to permit diffusion of oxygen and nutrients in depth, rapid and physiological colonization by cultured cells, production of extra-cellular matrix while biodegradation occurs. An appropriate evaluation of these properties allows to better foresee biological phenomena and cell behavior during tissue development on the scaffold, producing a more correct use of it and facilitating the identification of its possible fields of application (80), (101).

Histology and SEM based imaging is not practical to characterize 3D porosity or to model flow dynamics in porous scaffolds since images are only available in 2D slices and these methods involve destructive sample

preparation, are artifact prone and time consuming. While in recent years there are a number of publications on the use of microCT to characterize scaffold materials these were often carried out on hydroxyapatite or ceramics based materials, which have good x-ray contrast. However unstained bioscaffolds comprising soft materials such as collagen membranes and cross-linked gelatin materials used in this study often faced poor contrast and resolution limitations with conventional microCTs. In the current work we have used a novel microCT with very high contrast and which is capable of imaging these scaffolds at sampling sizes of several millimeter diameters at submicron resolution. With resolution close to histology or SEMs and coupled with the 3D volumetric data set, this non invasive tomography technique can more accurately characterize porosity and model flow dynamics in the range of scaffolds we are currently fabricating.

For this study we used: *i)* an already commercially available bi-layer membrane composed of type I and type III porcine collagen, bioresorbable within 4÷6 months, consisting of a top compact layer containing an inner porous and fibrous layer (Fig. 45 A-B-C) (146); *ii)* an experimental genepin cross-linked gelatin scaffold for chondrocyte culture and articular cartilage tissue engineering (Fig. 45 D-E-F); *iii)* an experimental GPMTS cross-linked gelatin scaffold for chondrocyte culture and articular cartilage tissue engineering.

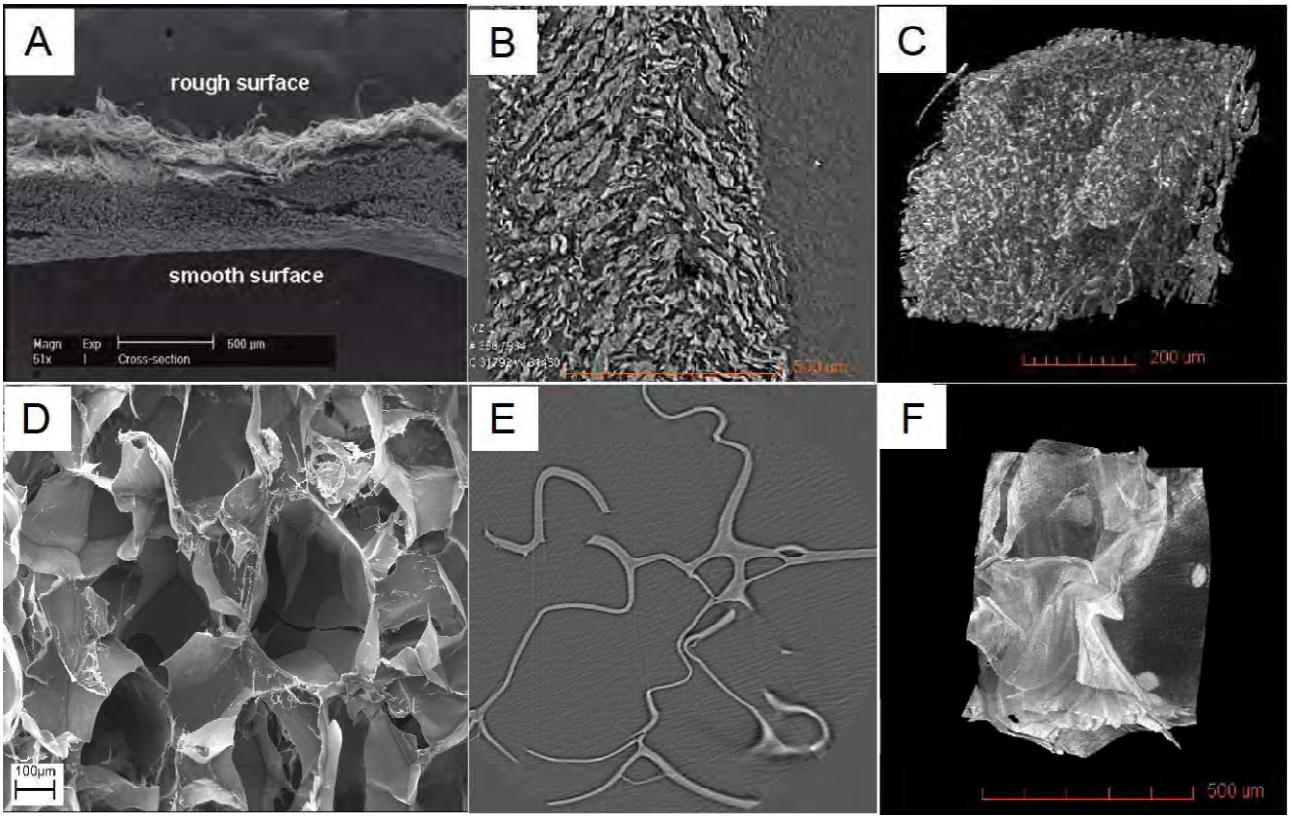


Fig. 45: A) SEM image (147) of collagen membrane, B) 2D CT slice and C) 3D rendered volume D) SEM image (100x) of GPMTS cross-linked gelatin scaffold E) 2D CT slice and F) 3D rendered volume.

Submicron tomography was carried out with Model MicroXCT (Xradia, Concord, CA, USA), operated at 40 kV energy with *PhaseEnhanced* detector for high contrast imaging of soft material without contrast agent (Fig. 46) (148). Image segmentation and porosity modeling was made by Amira©, whereas fluid dynamic modeling was carried out from the µCT datasets by first discretizing the volume into meshes and then solving the Navier–Stokes equations using a commercial computational fluid dynamics (CFD) package.

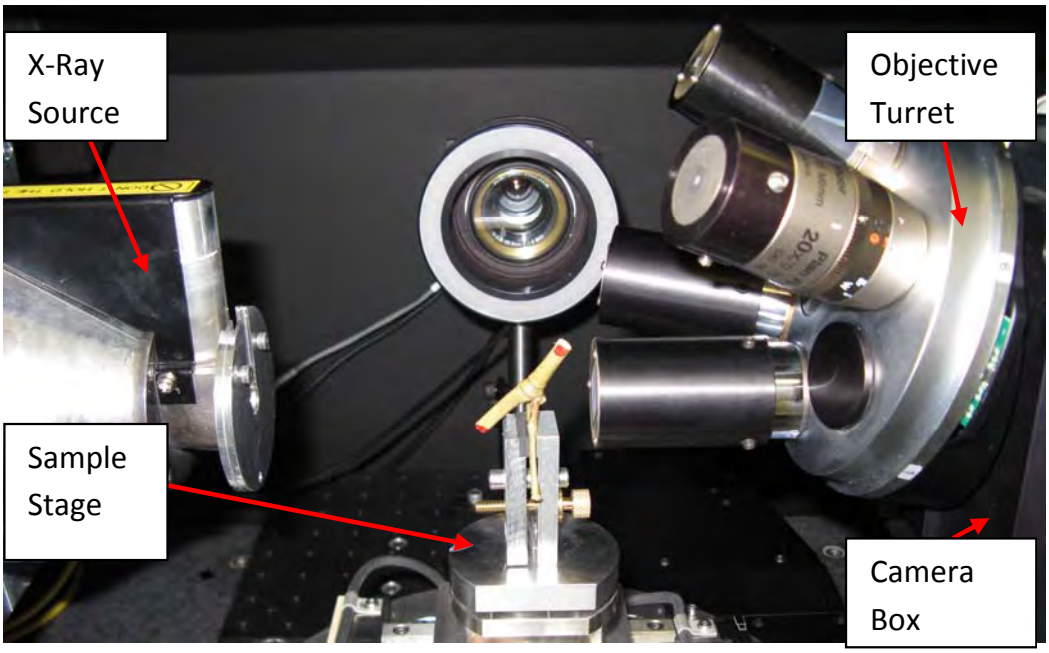


Fig. 46: Machine sample setup and layout example.

Fig. 47 and Fig. 48 show more detailed renderings of the scaffolds analyzed.

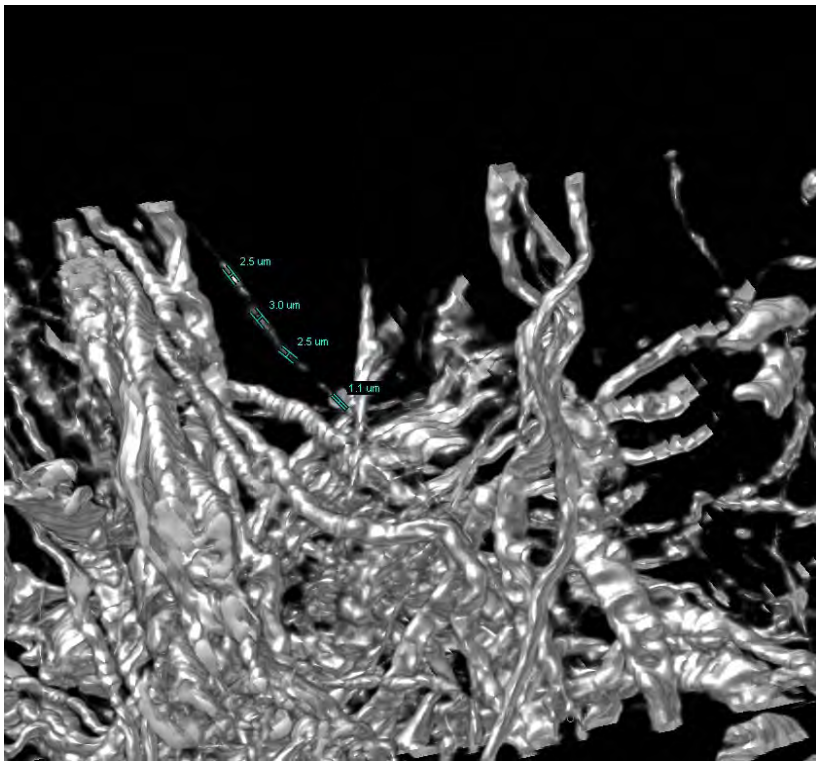


Fig. 47: Additional 3D Volume renderings of collagen membrane (147). Images are cropped and measured at different fibres. 20X objective at 0.967µm pixel resolution.

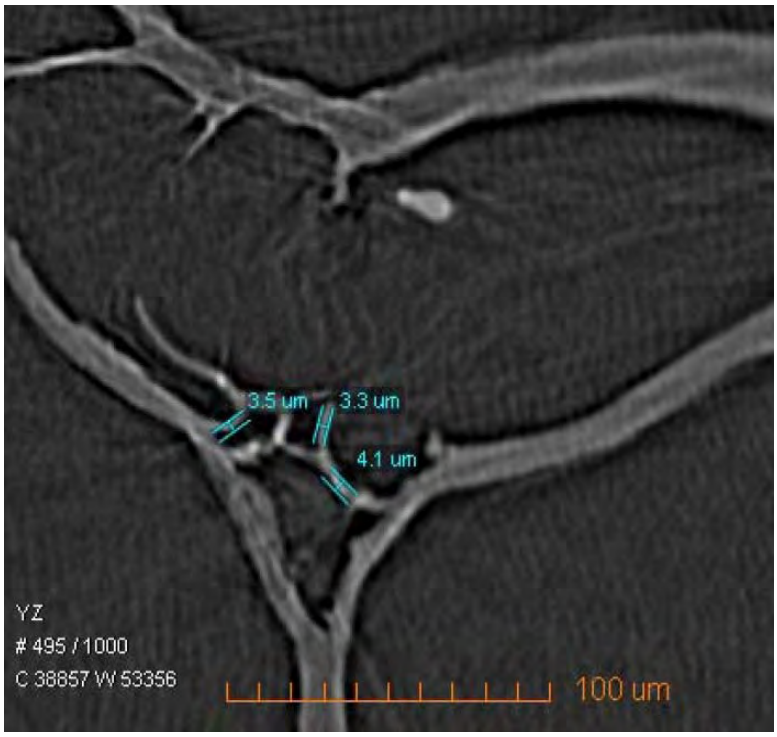


Fig. 48: A 2D YZ Plane virtual histology slices of sample 3 displays a zoomed in view of genepin cross-linked gelatin scaffold. 40X objective at 0.575µm pixel resolution.

The CFD simulation was carried out, following the steps shown in Fig. 49, using a flat velocity profile calculated for Reynolds number equal to 1 at the upper surface, no slip condition at the lateral surfaces and zero outlet pressure (at the lower surface).

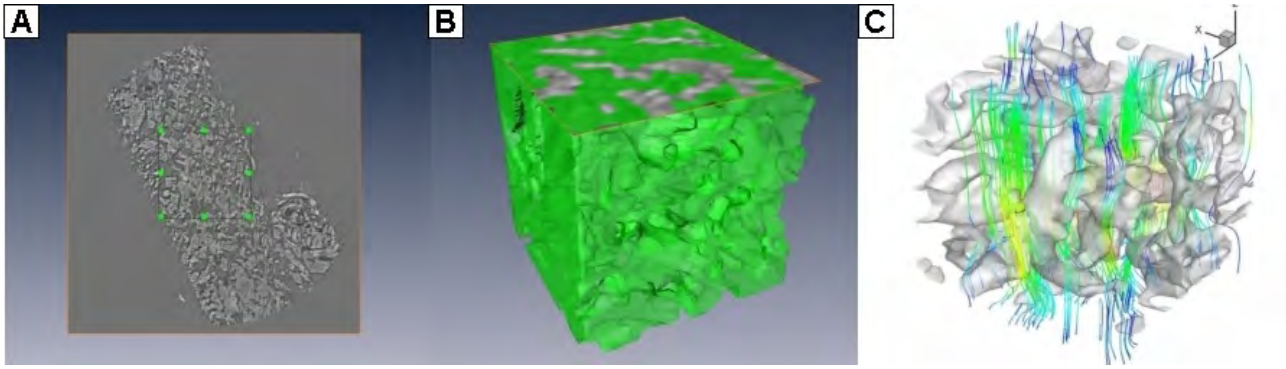


Fig. 49: The steps for analyzing computational fluid dynamics: (A-B) importing 2D slices into a segmentation package (Amira) in order (i) to distinguish the interconnected porosity from the wall by a thresholding algorithm; (ii) to apply the boundary conditions; (iii) to mesh and export the fluid (pore) regions in the correct format for the CFD solver. The CFD simulation (C) was performed on smaller ‘‘child” volumes (i.e. a sub-region extracted from the full 3D reconstructed µCT dataset).

This study demonstrated the applicability of novel microCT for the non-destructive quantification of several key characteristics of three different scaffolds, specifically: pore size distribution, interconnect size distribution and flow. The use of the novel microCT proposed in this study improves the efficacy of the computational methods by supplying higher resolution and contrast and taking to a more precise numerical description of the internal edges of the scaffolds under investigation, even in absence of staining.

1.3.2. Using lacunarity to characterize pore distribution in scaffolds

The performance of scaffolds for cells growth is largely influenced by their physical properties. In particular, as it has been stated before, porosity plays a major role. However, the characterization of the spatial

distribution of pores in irregular scaffolds is a challenging task, when the pores distribution is not homogeneous, with pores either clustered or dispersed (149). So, lacunarity has been proposed as a metric suitable for the characterization of the spatial distribution of pores in scaffolds for tissue engineering.

Already mentioned submicron resolution microCT images of Chondro-Gide® were used (Fig. 45 A-B-C). From them, synthetic images with the same porosity as the Chondro-Gide®, but with different spatial distributions of pores, were generated. On both recorded and synthetic images, lacunarity was calculated, in order to measure the spatial distribution of pores. Lacunarity analysis is a multi-scaled method of determining the texture associated with patterns of spatial dispersion. Lacunarity provides an analysis of scaffold images in terms of (*i*) the overall fraction covered by the attribute of interest, (*ii*) the presence and scale of randomness and (*iii*) the existence of hierarchical structure. Technically, to calculate lacunarity, from the Chondro-Gide® scaffold 2D CT slice, a binary map was created where each grid cell was denoted with zero (black cells, solids) or one (white cells, pores) (Fig. 50).

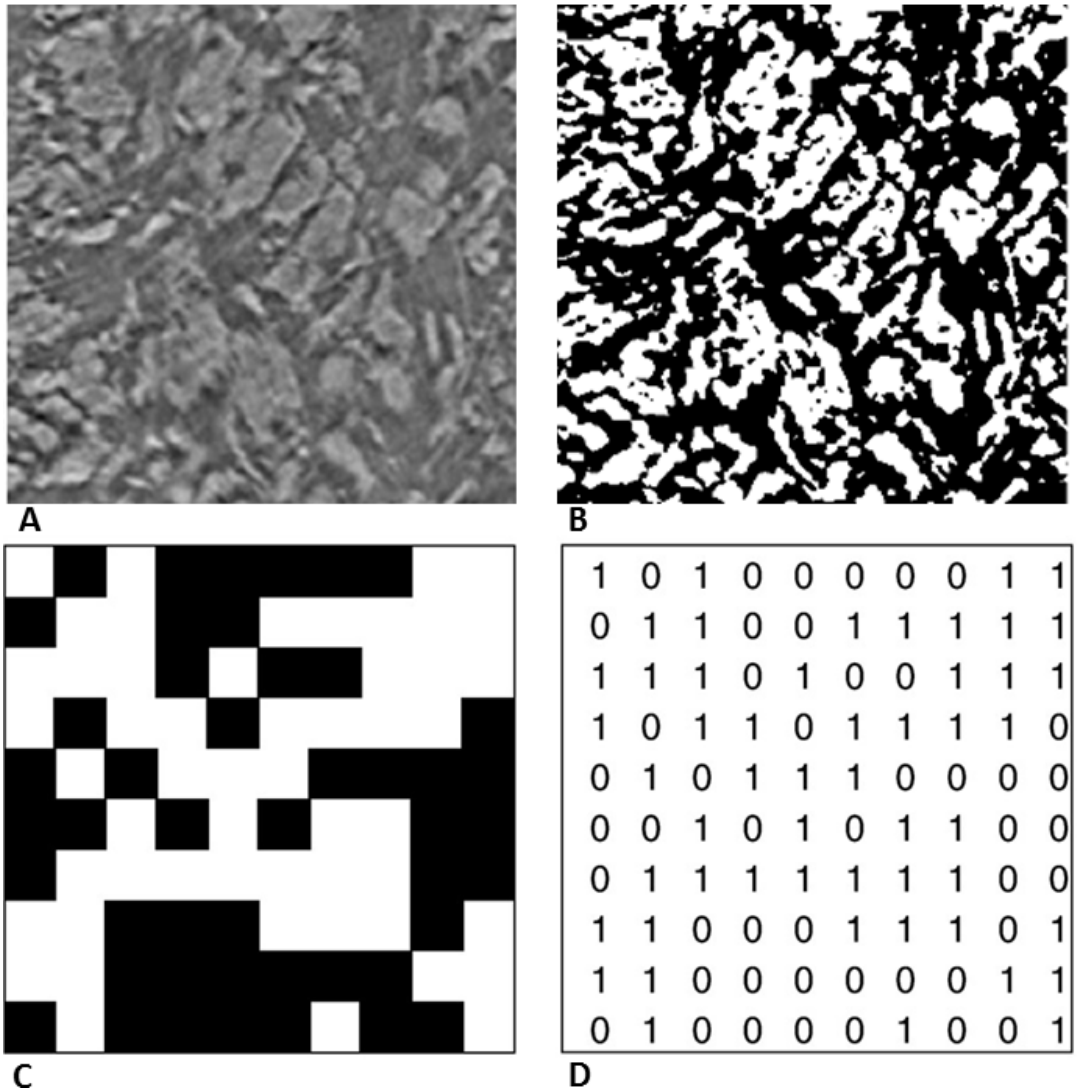


Fig. 50: Binarization of a Chondro-Gide® scaffold 2D CT slice (A-B) ; a synthetic random black (solid) and white (pore) map (C) and a binary representation of the same map (D).

Over each binary map obtained as at the previous step the LAC was finally calculated applying the 'gliding box' protocol (150) as follows: (*i*) a m*m box was superimposed to the map; (*ii*) starting from the upper left-hand corner, the box was moved one unit to the right (with a unit corresponding to the grid cell size)

and the number of zero and one grid cell contained within the box was counted (151); (*iii*) the box was shift down one grid cell size when the end of a row was reached and the process was repeated until the box was moved over all parts of the map; (*iv*) a frequency distribution of the number of the zero or one grid cell within the box was obtained and converted to a probability distribution. Lacunarity was finally calculated as

$$LAC = \sum_{p} \frac{p^2 P(p,m)}{\left(\sum_p pP(p,m)\right)^2} \qquad \textbf{Eqn. 56}$$

where *p* is the number of pore cells in a gliding window whose size is *m*, and *P(p,m)* is the probability function of the pore distribution.

We observed that in scaffolds with the same macroscopic porosity value, the greater the degree of pore clusterization, the higher the value of lacunarity. In fact, in synthetic scaffold images with porosity equal to 50% and with high clusterization, lacunarity is 1.83 (Fig. 51 B), while in the presence of dispersed pores lacunarity is reduced up to about 25% (Fig. 51 A). Interestingly, we conjecture that Chondro-Gide® is characterized by dispersed pores, because we calculated lacunarity equal to 1.38 on its microCT images (Fig. 51 C).

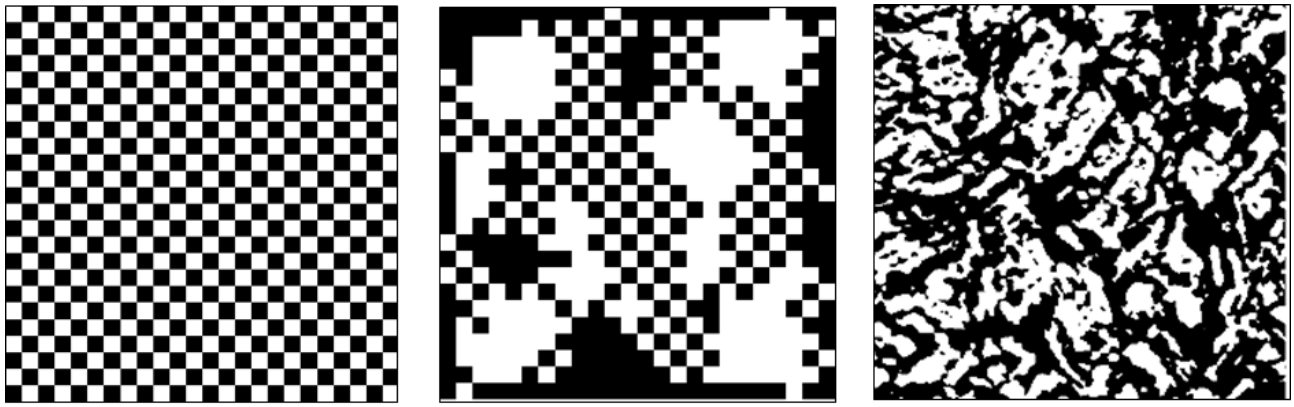


Fig. 51: Synthetic scaffold images with porosity equal to 50% with dispersed pores (A) and high clusterization (B); Chondro-Gide® binary slice (C).

Our findings clearly show that lacunarity can be a powerful tool for scaffolds properties characterization. In the future, pore clusterization will be analyzed to get insight into its influence on physical parameters such as permeability.

2. Basic research on bioreactors

The manufacture of 3D in vitro substitutes requires not only a biological source (proliferating cells with a precise phenotype), but also the development of new technological strategies of culture, addressed at reaching cellular densities higher than in traditional 2D cultures (in the order of 10^6 cells/cm3). In this perspective, tissue engineering has abandoned the use of traditional Petri dishes and 2D cell cultures, developing new technological tools for 3D cultivation: the bioreactors. Bioreactors are sterile apparatuses, that mimic physiological conditions, through the maintenance of physiological temperature and appropriate concentrations of metabolites and gases in order to support the growth of bio-artificial tissues. Bioreactors also allow the systematic study of the developing three-dimensional tissue in response to diverse chemical and physical stimuli. All cells under culture are able to sense and develop forces, thanks to their cytoskeleton, and modify morphology, differentiation capability and gene expression, in response to specific culture conditions. This fact is more important for cells with structural functions, as those of muscular, skeletal, and cartilaginous tissues. In physiological conditions, cells with structural roles are subjected to mechanical cyclic solicitations, whose defection brings to severe invalidating pathologies, as degeneration of join cartilage, bone fracture and myocardium infarction. Different types of bioreactors permit the contemporary culture of more than one biological patch, where each sample is subjected to external mechanical loads (gravity and movements), as well as internal forces (haemodynamic stresses and cells contraction). The specimens can be exposed to different stimuli and analyzed separately from the others.

2.1. Identification of stimuli promoting tissue development

In the biological world, a wide variety of different shapes and body profiles can be found. These natural phenomena have long been the focus of physical biologists (152). The intriguing question of how cells of only 10-40 µm in diameter can assemble and reproduce the shape of an organism that is meters in size has still not been answered. It is also not understood how cells can recognize their spatial position within such multicellular systems, even less is known about how they arrive at their destination within an organism. It is, however, known that cellular assemblies design and create the complex structure and morphology of different tissues during development. Moreover, extracellular matrices and neighbouring cells play an essential role in generating the major signals used by single cells to establish and maintain their shape and function (Fig. 52). On the one hand, single cells have to communicate with the biochemistry of their environment, such as the chemical nature of the extracellular matrix. On the other hand, they are also capable of sensing physical signals in their surroundings, such as forces and electrical stimuli. After sensing the signals, cells must respond appropriately to them, over time, in order to function properly.

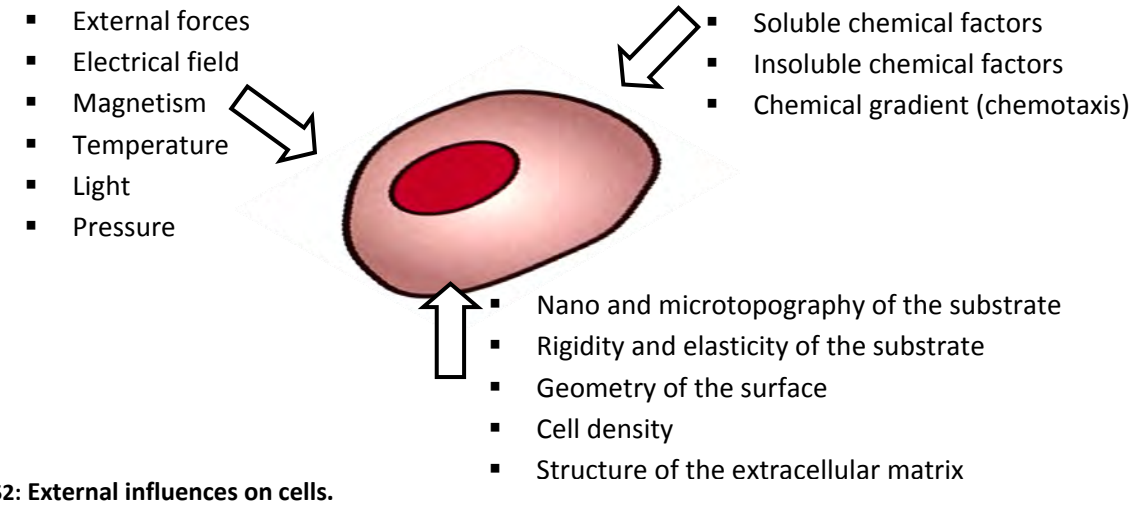


Fig. 52: External influences on cells.

Concerning the mechanical stimuli, from a tissue-engineering perspective, subjecting immature tissues to physiological loading could be a way to acquire the necessary structural and functional characteristics, and to enhance the transport of nutrients to cells within the constructs by inducing convection. In most cases, much remains to be learned about the exact nature, levels and regime of application of mechanical signals (153).

Instead, for the electrical stimulation, there are two possible mechanisms: via electrical gradients or via electrically inducted contraction, and the data presented in the literature, as well as data from 2D studies, suggest the latter. Interestingly, tissues engineered with the application of electrical field stimulation resemble in many aspects those engineered with mechanical stimulation. Electrical stimulation induced hyperpolarization at the anode end of the cell and depolarization at the cathode end of the cell, such that the cells aligned with the electrical field lines were subjected to the largest voltage difference and were likely the first ones to generate action potentials, contract, and couple with other cells via processes at the cells' ends (153).

However, the effects of different electrical and mechanical stimulation patterns on the activation of cell differentiation pathways are yet poorly understood, but the right application of these stimuli to high-density cultures of cells is an excellent strategy for engineering functional tissue constructs.

2.1.1. Influence of mechanical stimuli on tissue development

All cells in the body are subjected to mechanical forces that are either self-generated or originate from the environment. Depending on their location within the body, cells may be selectively exposed to various forces such as pressure, fluid shear stress, stretch, and compression.

Conventional cell culture techniques grow cells under static conditions; in large-scale bioreactors (e.g., fluidized bed reactors, spinner flasks, rotating vessels, and perfused vessels), flow and mixing patterns are introduced merely to enhance spatially uniform cell distributions on three-dimensional scaffolds and provide efficient mass transfer to the growing tissues. As the significance of externally applied mechanical forces in maintaining appropriate cell physiology has come into the light, tissue engineers have incorporated mechanical stresses into bioreactor design and found that physiological loading has positive effects on growing cells/tissues *in vitro* and plays a significant role in normal tissue homeostasis and remodeling. For example, gravitational compressive forces control bone deposition, mechanical loads on skeletal muscle determine muscle mass, and blood flow-associated mechanical forces regulate the homeostasis of vascular walls. In addition, increasing fluid shear forces significantly increases the mineral deposition by rat marrow stromal osteoblasts in a three-dimensional titanium fiber mesh scaffold, and the application of cyclic stretch to vascular smooth muscle cells (SMCs) cultured in collagen gels can help maintain the contractile phenotype of SMCs, align them in the correct physiological orientation, and improve the mechanical properties of cell–gel composites. In the context of tissue-engineered cartilage, researchers have found that artificial cartilage grown under cyclic compressive loading has superior biochemical compositions and material properties than those grown statically. Furthermore, cyclic compression can promote the chondrogenesis of rabbit bone-marrow mesenchymal stem cells (MSCs) by inducing the synthesis of transforming growth factor (TGF-β1), which then stimulates the MSCs to differentiate into chondrocytes (154).

All external forces that impinge on cells are imposed on a dynamic backdrop of various internally generated forces necessary for carrying out fundamental cellular events (e.g., cell division and migration). When cells sense a change in their net external loading, they actively alter their internal forces to counteract external

forces. There is growing recognition that the balance between internally generated forces and externally applied forces is a key determinant of cell fate (154). Moreover, many tissues in the body are subjected to internal and external mechanical stimuli and researchers now discovered the last years the importance of forces in various biological processes, particularly in tissue engineering or disease. For example, patterns of cellular growth can generate forces that help to bring tissue into their specific forms (155). It was shown that forces generated by tissue itself play a major role as a possible feedback regulator of tissue growth (156). Furthermore, for the success of fracture repair, forces are of immense importance (157). Mechanical load is an important signal which serves to regulate the growth of bones. Early studies in the 1970 showed that disuse of the multiskeletal system influences the growth and stability of bones. In that connection, Uthoff et al. (158) performed experiments, comparing normally used and disused bones of 57 young and old dogs. They found that a disuse of bone destabilize it, and changes the morphology, resulting in a loss of bone mass.

Similar effects can be seen in the engineering and development of tissue-engineered tendons. In studies of Calve et al. (159), the stress-strain curve of the natural tendon from a chick embryo and a tissue-engineered tendon which was not subjected to mechanical load were compared. They showed that both tendons displayed the same characteristic; however, the mechanical properties of the constructed tendon were significantly lower, in an order of magnitude, than those of natural tendon. This means that when designing artificial tissues, we should keep clearly in mind that mechanical aspects, like external or internal forces, play a critical role in their proper functioning (160). Furthermore, new studies showed that the age of cells influences mechanical properties and signaling of cells. Studies of Blough *et al.* displayed that rat aortic tissues becomes stiffer with age (161) and studies of Berdyyeva where they measured the stiffness of endothelia cells by means of atomic force microscopy corroborate these results (162). Together with studies showing that changes in the extracellular matrix as well as in the cell itself as it ages influence the complex mechanism of mechanotransduction and mechanophysiology (163), we can expect, that the cellular reaction on mechanical signals differs with age.

As previously mentioned, mechanical effects can influence several biological processes (164). At the single cell level, force can influence cellular functions such as gene expression, proliferation, apoptosis, differentiation, reorganization of internal cell structures, or reorganization of the entire cell (Fig. 53). Force can initiate cell protrusion, alter motility, and affect the metabolic reactions that regulate cell function, cell division or cell death. Examples are: (i) dysfunction of lymphocytes at near-zero gravity (165); (ii) force-dependent acceleration of axonal elongation in neurons (166; 167); (iii) forcedependent changes in the transcription of cytoskeletal proteins in osteoblasts and other cell types (168); (iv) altered transcription in endothelial cells subjected to flow (169); and (v) forcedependent changes in the morphology and orientation of cells and their cytoskeletons (170). In addition to externally imposed forces, cells also exert internally generated forces on the matrix on which they adhere. Some types of cells can behave as a kind of detector of material stiffness, changing their structure, motility and growth as they sense the mechanical properties of their surroundings (171).

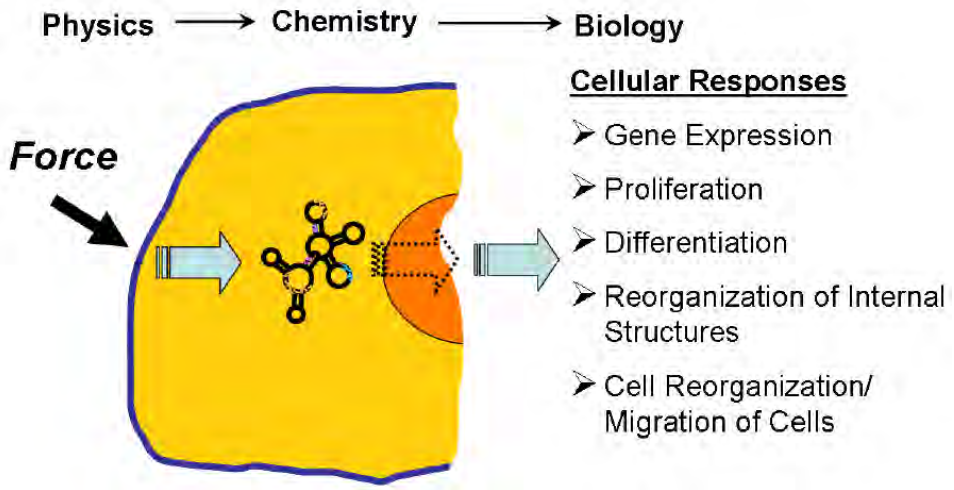


Fig. 53: Mechanical forces in cell biology and their impact on cellular reactions. External forces must be translated via chemistry to a biological response (164).

Mechanical stimuli have been reported to have profound effects also on primary cardiomyocytes, although its effects on the cardiomyogenic differentiation of stem cells have not yet been investigated. Application of mechanical stimuli can either be in the form of passive mechanical stretch loading or active contractile stretch stimulation. With neonatal cardiomyocytes, passive mechanical stretch loading has been reported to upregulate myosin heavy chain expression and induced cardiomyocyte organization into parallel arrays of rod-shaped cells. Adult cardiomyocytes were however weakly stimulated by passive mechanical load . Wada *et al.* reported that for adult cardiomyocytes, contractile mechanical stretching provided a much stronger stimulus for protein synthesis, as compared to passive mechanical load. With neonatal cardiomyocytes, contractile mechanical stimulation induced secretion of growth promoting factors, as well as upregulation of connexin-43 and myosin heavy chain. Both of these are markers of cardiomyocyte maturity. Kada *et al.* reported that embryonic rat cardiomyocytes subjected to cyclical stretching resulted in parallel orientation of cardiomyocytes and their intracellular myofibrils. When cyclical stretch stimulation was prolonged, myofibrils that orientated perpendicular to the stretch direction emerged. To date, the pathway by which mechanical stimuli is transduced into a biochemical signal that initiate cardiomyogenic differentiation, has not yet been elucidated. Nevertheless, Schluter and Piper have provided a hypothetical mechanism, in which mechanical forces are transmitted through costameres that are coupled to integrin signaling molecules (172).

Cells from different tissues respond to varying types and levels of stress (force per unit area). For example, cartilage typically experiences stresses of 20 MPa. The chondrocytes within the cartilage can modify the expression of glycosaminoglycan or other constituents in response to these forces (173). In a similar manner, osteocytes in bone respond to mechanical stress. Endothelial cells in contrast, respond to a shear stress of less than 1 Pa (174) and neutrophile granulocytes react to even lesser amounts of shear stress (175). It is not only the strength of the applied forces, but also the time course or dynamic changes of the applied force that constitute crucial factors in the cellular response to stress. For example, endothelial cells in blood vessels respond to changes to turbulent stress, rather than to a specific magnitude of stress (169). Recent findings demonstrated, that the loading rate of the force onto the cell, and the matrix stiffening by the cellular activity can have a crucial impact on the cell itself (176). For example, the application of defined mechanical stimuli to cultured cardiac fibroblasts has been associated with ECM gene expression and growth factor production, release and/or bioactivity. Dynamic stretching of cardiac fibroblasts caused the activation of β1-integrin dependent ERK and JNK pathways, as well as the expression of collagen III,

fibronectin and TGF-β1. Hence, the stretch-induced response is being transmitted, for instance, to cardiomyocytes through autocrine or paracrine signaling and ECM remodeling. Changes in ECM structure and mechanics were suggested to alter the balance of forces that are transferred across the cell surface adhesion receptors that line the ECM to the internal supporting framework of the cell, the cytoskeleton (177). Both mechanical sensing and the integration of different mechanical signals at different locations and times, occur through complex signaling pathways and target proteins that activate a programmed response to properly form, shape and influence cells and tissues (176). In particular, evidence of local mechanical control of tissue patterning awaits development of new methods for applying controlled stresses for real-time readout of cellular response.

2.1.2. Influence of electrical stimuli on tissue development

Electrical stimulation (ES) has been widely applied in experimental and clinical settings in order to achieve beneficial effects on the clinical care process, to facilitate cellular adaptation to an experimental intervention or to elucidate cellular responses. Submitting a tissue construct to a biomimetic electrical stimulation during cultivation *in vitro* improves its structural and functional properties, and/or *in vivo*, after implantation of the construct, enhances its integration with host tissue and increases cell survival and functionality. The methods are particularly useful for the production of bioartificial equivalents and/or the repair and replacement of native tissues that contain electrically excitable cells and are subject to electrical stimulation in vivo, such as, for example, cardiac muscle tissue, striated skeletal muscle tissue, smooth muscle tissue, bone, vasculature, and nerve tissue (178). Unfortunately, in spite of ES-induced effects are well-documented, the mechanisms underlying ES-induced effects at the cellular as well as the molecular level remain to a large extent unknown. ES has been shown to have a beneficial effect on the wound healing process, a fundamental impact on regeneration of injured tissues and cellular maturation. There is also evidence that ES can distinctly affect the immunologic response. It was shown in an animal study that transcutaneous needle stimulation causes a reversal of stress-induced suppression of antibody production. Thus, ES can bring about a variety of functional and structural changes in the cells. However, the mechanisms underlying ES-induced cellular changes are not thoroughly investigated. Based on the fact that ES exerts diverse effects, it is likely that treatments with different kinds of ES on different tissues share a common molecular mechanism (179).

As previously mentioned, the relationship between physical forces and bone biology has been recognized since the early 1800s. Mechanical forces (compression, distraction, and shear), electrical forces, magnetic forces, and ultrasonic waves have all been found to exert some level of effect on bone growth and healing. Electrical stimulation of bone has been touted as an effective and noninvasive method for enhancing bone healing, and treating fracture nonunion. Unfortunately, clinical evidence for the efficacy of electrical stimulation is limited. A recent meta-analysis by Mollon *et al.* could only identify four randomized controlled trials evaluating the clinical use of electrical stimulation to treat delayed union and nonunion of fractures (180). Despite the lack of clinical evidence, many *in vitro* and *in vivo* studies demonstrate the usefulness of electrical stimulation in bone healing at a cellular level. In fact, direct electrical current (DC), capacitive coupling (CC), and inductive coupling (IC) have been studied as potential techniques to enhance fracture healing through the proliferation and differentiation of osteogenic cells (181). Examples are: (i) DNA production in cultured bone cells following CC stimulation (182); (ii) increase in healing tissue TGF-β1 levels in response to therapeutic IC fields (183); (iii) progenitor cells involved in enchondral bone formation showed upregulated differentiation, ECM synthesis and TGF-β1 expression (184); (iv) direct electrical current promoted healing of spinal fusion, ankle fusions and charcot foot reconstructions (185; 186; 187) ; and (v) the direct electrical current also results in increased proteoglycan and collagen synthesis. In

addition, hydrogen peroxide may stimulate macrophages to release vascular endothelial growth factor (VEGF), an angiogenic factor that is critical for osteogenesis (181; 188; 189).

Similar effects can be seen in the engineering of cardiac tissue-engineered constructs; development of their conductive and contractile properties is concurrent, with strong dependence on the initiation and duration of the applied electrical stimulation. The control of heart contractions is almost entirely self-contained. The propagation of electrical signals across specialized intracellular junctions produces mechanical contractions that pump blood forward. Orderly coupling between electrical pacing signals and macroscopic contractions is therefore crucial for the heart development and function (153). Groups of specialized cardiac myocytes (pacemakers), fastest of which are located in the sinoatrial node, drive periodic contractions of the heart. The majority of the cells in the myocardium are non-pacemaker cells and they respond to the electrical stimuli generated by pacemaker cells. Excitation of each cardiac myocyte is followed by an increase in the amount of cytoplasmic calcium that triggers mechanical contractions. The propagation of the electrical excitation through the tissue by ion currents in the extracellular and intracellular spaces results in synchronous contractions (177). Moreover, electrical stimulation has been shown to improve functional assembly of cardiomyocytes in vitro for cardiac tissue engineering (190).

For example, in studies of Radisic et al. (191), cell populations isolated from neonatal rat heart ventricles are cultured on a biomaterial scaffold in a bioreactor (providing environmental control and the application of molecular and physical regulatory signals). To enhance functional cell assembly, they induced synchronous contractions of cultured cardiac constructs by applying electrical signals designed to mimic those orchestrating the synchronous contractions of cells in native heart. Over only 8 days in vitro, electrical field stimulation resulted in cell alignment and coupling, markedly increased the amplitude of synchronous construct contractions and resulted in a remarkable level of ultrastructural organization (192). Electrical stimulation also promoted cell differentiation and coupling, as evidenced by the presence of striations and gap junctions, and resulted in concurrent development of conductive and contractile properties of cardiac constructs (192; 191). Studies of Tandon *et al.*, where they used different type of electrodes (nanoporous carbon, stainless steel, titanium and titanium nitride electrodes) to enhance functional assembly of isolated heart cell populations corroborate these results.

Furthermore, similar data can be confirmed by the studies of Cannizzaro et al. (193), in fact they observed that the application of electrical stimulation to cardiac constructs markedly enhanced the contractile behavior. After 8d of culture, the amplitude of contractions was sevenfold higher in stimulated than in nonstimulated constructs, a result of the progressive increase with the duration of culture. Engineered constructs that were electrically stimulated during culture demonstrate a remarkable level of ultrastructural differentiation, comparable in several respects with that of native myocardium (Fig. 54).

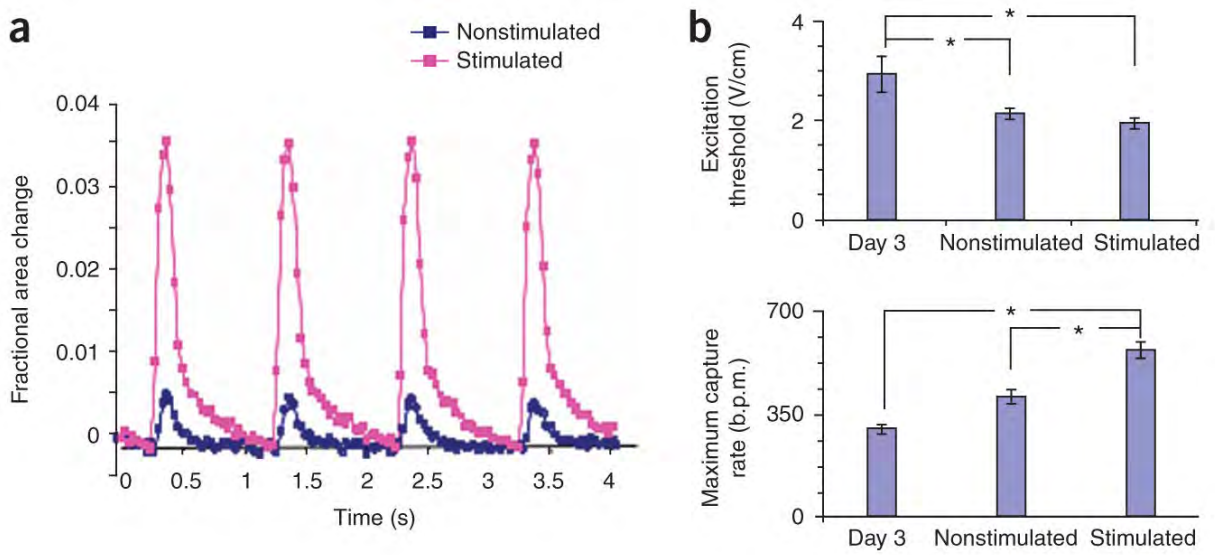


Fig. 54: Orderly excitation–contraction coupling enhances functional assembly of myocardium in vitro. (a) Contraction amplitude (the fractional change in area of tissue during a contraction) increases with electrical stimulation. (b) Excitation threshold (electrical field voltage gradient that needs to be applied to induce synchronous contractions of cultured tissue constructs) and maximum capture rate (the maximum frequency at which tissue constructs can be induced to beat) significantly decreased and increased, respectively, with the application of electrical stimulation and with time in culture (193).

Myofibers align orthogonal to the direction of the electrodes and the collagen sponge scaffold consists of isotropic interconnected pores. In contrast, cells in nonstimulated constructs stayed round and expressed relatively low levels of cardiac markers. Electrical stimulation also increased the amounts of mitochondria and glycogen, and induced the formation of well-aligned registers of sarcomeres that closely resembled those in native myocardium, representing a hallmark of maturing cardiomyocytes. The volume fraction of sarcomeres in stimulated 8d constructs was indistinguishable from that measured for neonatal ventricles, in contrast to nonstimulated constructs with only scarce and poorly organized sarcomeres. Stimulated constructs also had well-developed intercalated disks and gap junctions (193).

Since the effects of different electrical stimulation patterns on the activation of cell differentiation pathways are poorly understood, the main aim of these work is to further analyze different electrical stimulations on cells differentiation.

It was shown that pulsed electric signals are implicated *in vivo* in the formation of the cardiac syncytium during the embryonic heart development. *In vitro* exposure of stem cells (SCs) to electrical pacing has been found to promote cardiomyogenic differentiation and to enhance functional coupling of the cells resulting in the formation of synchronously contractile tissue constructs.

Studies of Pavesi et al. (194), explored and compared the effects of patterns of electrical field stimulation on SC cardiac differentiation. They developed a bioreactor for electrical stimulation with features a chassis, including electrical wiring systems, equipped with housings for multiple culture chambers: this allowed for multiple simultaneous cell cultures with independent electrical stimulation patterns, also avoiding the risk of cross-contamination during the experiments. The electric field distribution within the 3D environment of

the bioreactor was evaluated using a finite-element technique-based software. They observed that, when a 8-V amplitude voltage is applied at the electrodes, the cell-seeded surface experiences a rather uniform 5 V/cm electric field, strongly aligned along the direction perpendicular to the electrodes axes (Fig. 55).

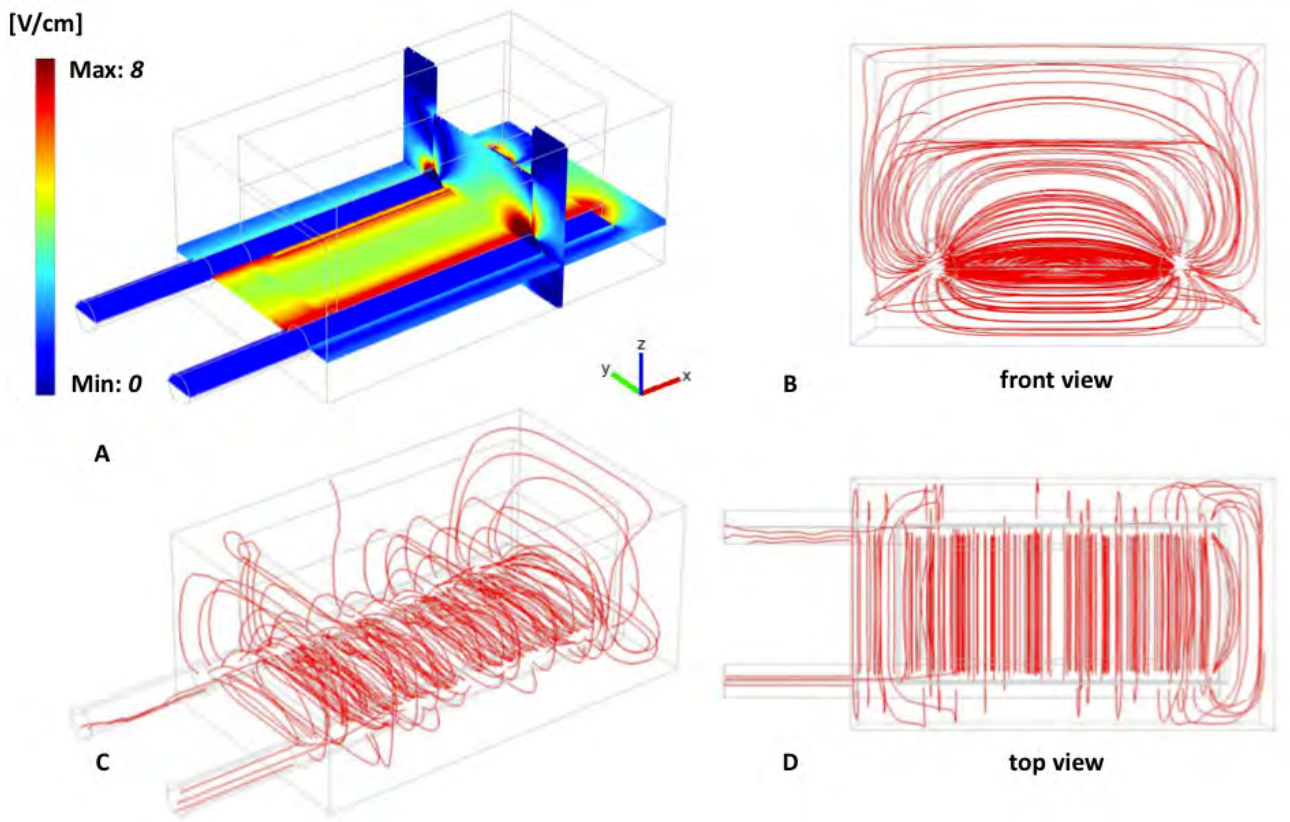


Fig. 55: Contours of magnitude of the modulus (A) and streamlines (B, C, D) of the electric field are plotted in the whole culture chamber and on the cell-seeded surface, where a quite-uniform 5-V/cm electric field is attained, strongly aligned along the x axis (194).

For the *in vitro* studies, spontaneously immortalized multipotent cells derived from mouse adipose tissue were dropwise seeded on glass slides housed within the chambers (3000 cells/glass slide). After 4 days of static culture, the electrical stimulation was started. Either square monophasic (2 ms, 1 Hz, 8V amplitude) and biphasic (2 ms, 1 Hz, ±4 V amplitude) pulses were applied to the cultured cells. Stimulation efficiency of the mono/biphasic pulses was determined by evaluating cell morphology, proliferation and expression of the Connexin 43 (Cx-43) cardiac marker, after a 3-day electrical stimulation period.

Preliminary *in vitro* tests (Fig. 56) demonstrated that cells exposed to a biphasic pulsed electric field express Cx-43 in the peripheral cell region, thus suggesting the tendency to form the cell-cell connections involved in myocardial electric propagation. Instead, expression of Cx-43 was poorly observed after monophasic stimulation, whereas Cx-43 was completely absent in not stimulated (control) samples (194).

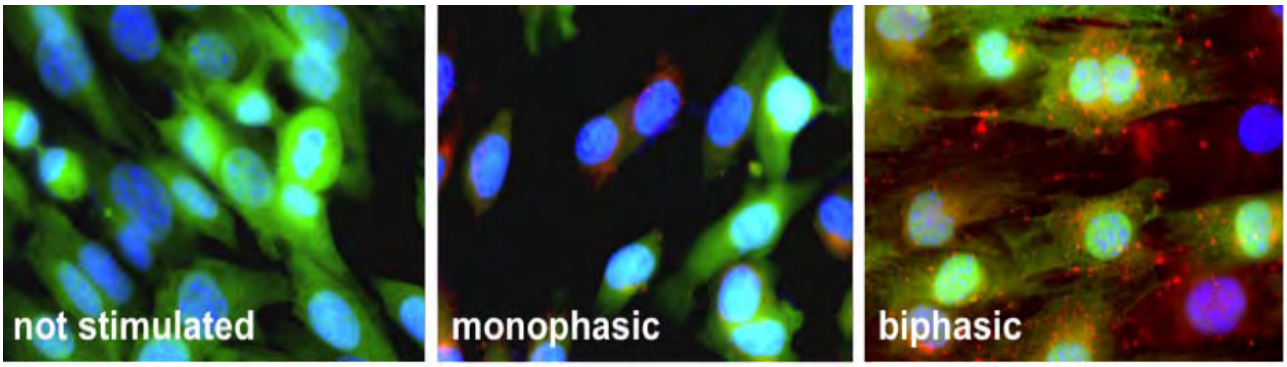


Fig. 56: GFP (green), DAPI (blue), Cx-43 (red) immunofluorescence staining of cultured cells at the end of a 3-day period of electrical stimulation (194).

2.2. Bioreactor general design requirements

In order to provide cells with the physiological stimuli, to analyze the cellular responses and measure key variables in the culture system, the bioreactor is made up of separate functional modules. Bioreactors therefore arise from the systematic assembly of individual components each of those has specific function: modulate culture condition (actuators) or monitor key parameters (sensors). Each module works in combination with the others. Particular attention must be addressed to the technology of assembled bioreactors for the culture of cardiac myocytes and mesenchymal stem cells, which can differentiate in osteoblasts, cardiac myocytes and skeletal tissue.

Each component of the device will be analized. We will deal with:

- Chambers, seals and latches;
- Pumps and fluids;
- Sensors;
- Control processes;
- Actuators;

The objective of the first part of this tractation is the depiction of the state-of-art of the technologies involved in the single bioreactor modules. For completeness of tractation, we also overview the materials employed, compatible with common sterilization processes. In the second part of the bioreactor design we define the best features of such a system, with particular attention to the cultivation of cardiovascular and mesenchimal cells.

In order to rationally design a bioreactor, we need to consider some general characteristics. As reported by Freed and Vunjak-Novakovic (195), a bioreactor should perform at least one of the following functions: create physiological conditions and allow cells to proliferate and differentiate as in vivo; enable cells to arrive at a uniform distribution within the scaffold; furnish desired concentrations of nutrients and remove metabolites and toxic products; provide efficient mass transfer; expose the tissue to physical stimuli resembling the physiological ones. These aspects can be taken into account more specifically if we consider the bioreactor as an assemble of functional modules. Each of those modules provides a specific condition for the maintenance of the proper conditions in a cell culture: sensors, actuators, control system, chamber geometry and materials, as well as pumps and fluid flow conditions need to be accurately evaluated in the design of a bioreactor for tissue engineering.

Sensoring apparatus, control systems and actuator devices work in combination. In fact, the growth of cells is supported by a precise control of the in vitro environment, aimed at maintaining temperature, pH values, media flow rate, nutrient and oxygen concentrations within close physiological limits.

Tissue culture is a non-steady state process and some parameters continually change. Currently it is not possible to easily measure all of these variables on-line, especially the biological factors. Thus one of the requirements for the future is to develop sensors for on-line measurement, or alternatively to remove samples for fast, near-on line analysis. Also, the visual analysis of the culture is a key control of the tissue ingrowths: the bioreactor chamber should be transparent for allowing microscopical observations of the developing tissue and should be designed in order to prevent the entry of infecting microorganisms, which could affect the values of the culture parametres. In this perspective, the choice of the culture chamber, seals and latches is a critical task for bioreactor design. As suggested by Sinclair and Ashley (196), the requirement of sterility can also be further broken down into the following: choice of a material compatible with common sterilization processes and pre-sterilization of the equipment and manteinance of sterility during culture.

Another part of relevance is the pump module, enabling gas exchange and specific fluid flow conditions. Cells need to be exposed to a physiological environment, where fluid flow resembles rates, shear modulus and chemical composition of those in vivo. Indeed, the exchange of gases within the bioreactor system is associated with the provision of nutrients to cells and the maintenance of pH in the chamber.

2.2.1. Chambers, seals and latches

For cell culture, the choice of the geometry of the chamber, of materials, as well as the sterilization procedures employed are specific of the bioreactor type and the culture conditions. Some examples are illustrated in the following paragraphs, where a brief analysis of the previously designed chambers for tissue engineering constructs is developed. In particular, we will address: chambers for the cultivation of several constructs in parallel; chambers obtained via different machining techniques; chambers with minimal angular geometries; chambers designed for flow perfusion systems and chambers designed for organ and complex tissue culture.

An example of chamber for the cultivation of multiple specimens in parallel has been introduced by Engelmayr et al. (197) (Fig. 57). They addressed their experimental studies to the development of tissue engineered heart valves, employing a type of bioreactor consisting of two identical chambers. The chambers were machined from polysulfone, chosen for its excellent thermal and chemical stability, and abrasion-resistant acrylic, which provides good optical transparency. Neoprene gaskets were fitted between all critical joints in order to reduce the possibility of microbial contamination, and the bioreactor was assembled using 18–8 stainless-steel screws (McMaster-Carr Supply Co.,Cleveland, OH). Each of the two identical chambers (127mm x 101.6 mm) contains 6 culture wells (25.4mm diameter, 16mm deep, (C)). Situated within each well are four stainless-steel ‘‘stationary posts’’ arranged orthogonally around a central channel in the floor of the culture well (1.9mm deep). The device can thus accommodate a total of 12 rectangular samples (maximum dimensions approximately 25mm x 7.5mm x 2 mm), with each sample being positioned between the four stationary posts, orthogonal to the central channel. The structural elements of the device can be cold gas sterilized by ethylene oxide, and the entire device is designed to be operated inside of a standard humidified incubator.

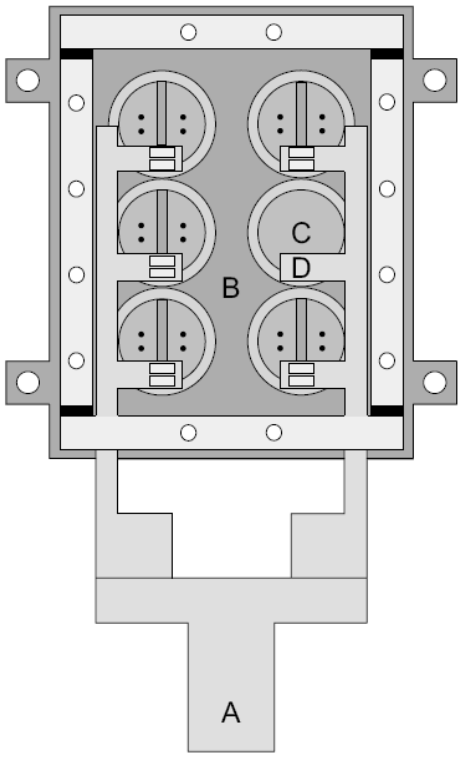


Fig. 57: Schematic diagram of a single chamber of the bioreactor. The arm connects to the cross-arm at (A), bifurcates before penetrating the wall of the chamber, and terminates in six fingers (D). Two ''flexure pins'' can be inserted through each finger, protruding into each culture well channel such as to bracket the center of a rectangular sample. The interior of the chamber (B) is protected from contamination by a lid (not shown) (197).

Another type of bioreactor system for the cultivation of several constructs in parallel and also composed of two chambers is the double-chamber bioreactor, proposed by Chang et al. (198). It consists of two tubular shape glass chambers, each of which has four branch tubes (medium inflow and outflow, oxygen ventilation, one surplus and reserved for other functions, where necessary, Fig. 58 and Fig. 59). The whole bioreactor is autoclavable. An aseptic ventilation filter is connected to one branch tube, one for each chamber under laminar flow. The chambers are separated by a silicone-rubber septum with multiple holes to hold the biphasic scaffold. Magnetic-bar stirring provides mixing of the medium and mechanical stimulation, such as shear stress for the engineered cartilage. The bioreactor has two independent medium-circulation systems and, thus, can be used to supply various types of culture media to support co-culture of different kinds of cells, or different induction media.

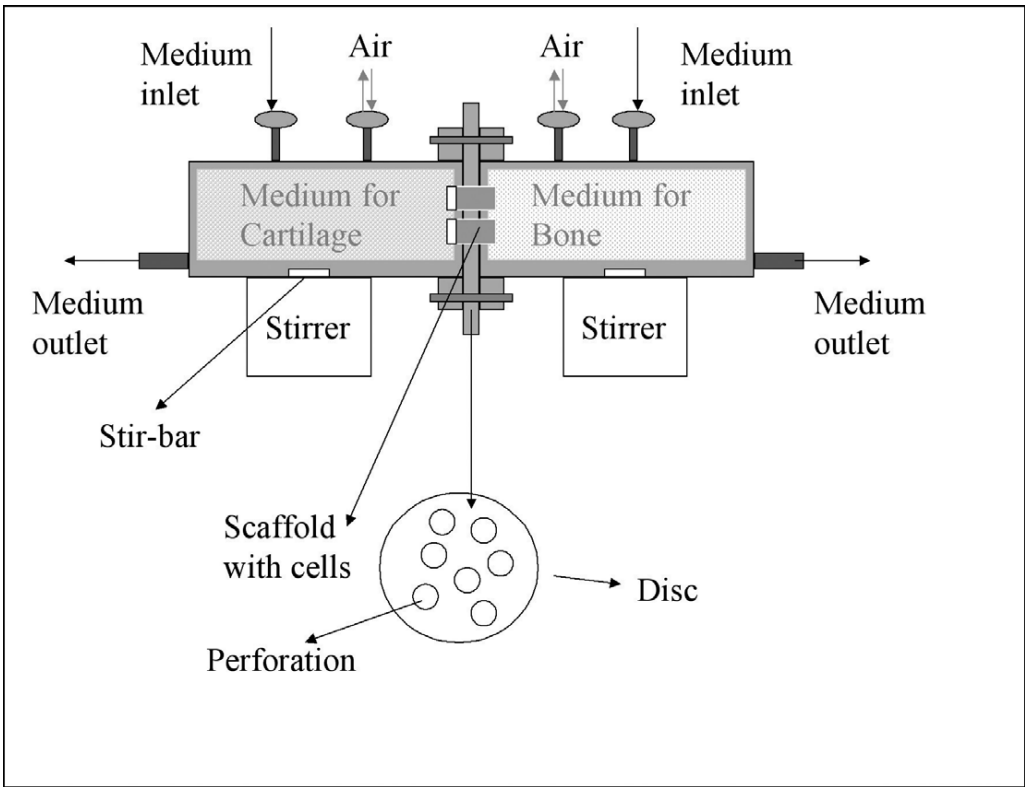


Fig. 58: Design of a double-chamber bioreactor: The double-chamber bioreactor consisted with two glass chambers. Each chamber contains three branch tubes for medium inflow, medium outflow and oxygen ventilation. The two chambers are separated with a silicon septum with multiple holes to hold the biphasic scaffold. Magnetic bar stirring provided the mechanical stimulation for the scaffold. The bioreactor contains two independent medium circulation systems (198).

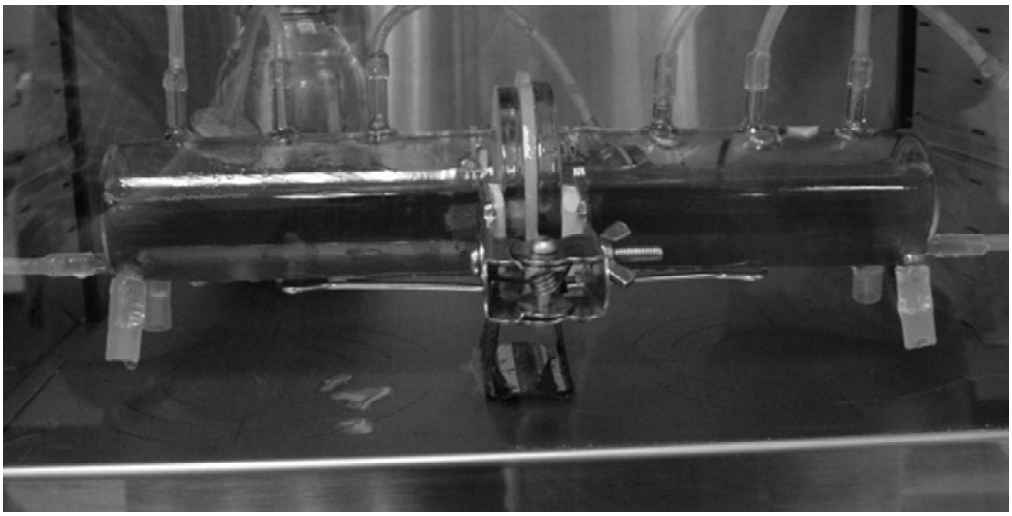


Fig. 59: Double chamber bioreactor in the incubator (198).

Other kinds of culture chambers have been designed for the cultivation of several constructs in parallel. The flow chamber proposed by Nagel-Heyel (199) enables the cultivation of six cartilage-carrier-constructs in parallel (Fig. 60).

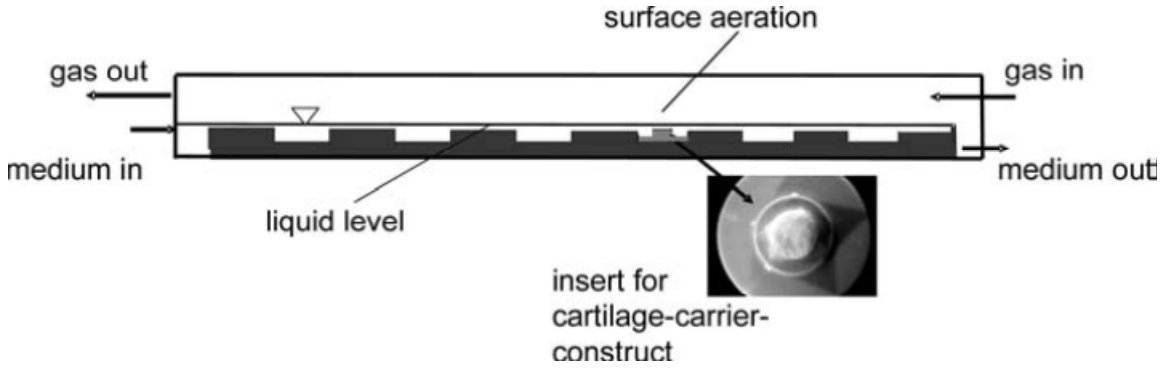


Fig. 60: schematic model of the chamber for the cultivation six cartilage-carrier-constructs in parallel (199).

The constructs can be placed in inserts, which can be first used during the preculture and then transferred to the flow chamber. A specific attribute of the flow-chamber is the counter current flow of medium and gas. The gas (air + 5% CO2, 5 L h-1) flows in the headspace of the flow chamber above the medium. The height of the medium layer above the constructs is kept below 3 mm by means of a small border, which creates an overflow. By this an optimal oxygen supply for all constructs can be achieved. The chamber is made of polyetheretherketon (PEEK) with the following dimensions: length 21 cm, width 9.5 cm, height 4 cm. Dimensions of the flow channel are: length of 18 cm, width 2 cm, height 2 cm. A glass plate is integrated in the top.

Another chamber for the cultivation of many constructs in parallel is that of the T-CUP bioreactor system (Fig. 61). The scaffold housing (chamber) is made to accommodate 6 scaffolds in the shape of 8mm diameter 4mm thick cylinders. In some experiments, the scaffold housing is in the form of a basket, in which granular porous scaffolds are irregularly compacted. The chamber of culture is fabricated in polytetrafluoroethylene (PTFE) and monted into a dual-upper reactor cup, made of stainless steel (SS316L). Internal surfaces are either coated with a 30-mm layer of fluoroethylene propylene (FEP) or electropolished to ensure that cells would not adhere to the bioreactor and to improve cleanability. FEP coating or electropolishing are selected after preliminary experiments made with other materials, which either do not prevent cell attachment (i.e., polycarbonate, polysulfone) or are progressively deformed by repeated autoclaving (i.e., PTFE, polyetheretherketone) (200).

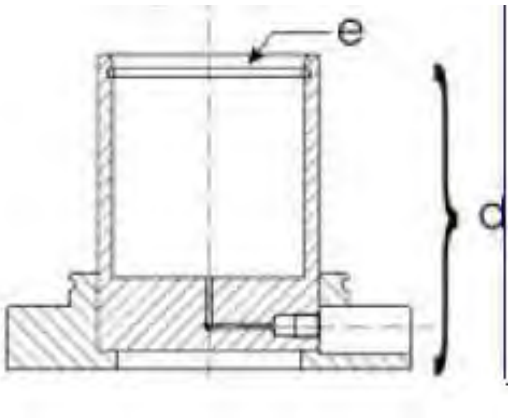


Fig. 61: The TCUP bioreactor system. The dual-upper cup is mounted over the lower cup, also made of stainless steel (part d), creating a closed chamber that was dynamically sealed with an O-ring (part e) (200).

Ininich et al. (201) used a bioreactor consisting of eight chambers, designed for dynamic compression of cartilage samples (Fig. 62).

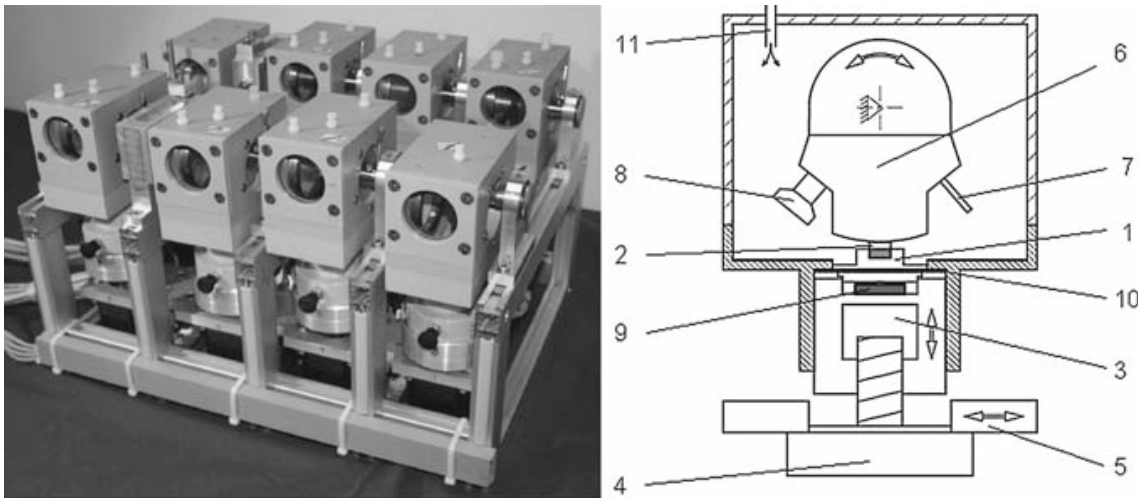


Fig. 62: Bioreactor with eight chambers for cultivation of eight cartilage samples under load application (201).

A single chamber bioreactor was designed for the dynamic loading and the fabrication of tissue engineered mitral valve chordae tendineae (Fig. 63 and Fig. 64) (202). These “one dimensional” structures need to be loaded only longitudinally, requiring a relatively simple chamber in the bioreactor system. The rectangular chamber is constructed from silicone rubber in the 100 mm stereolithography (SLA) culture dish similar to those used in the static loading bioreactor. SLA is a technique typically used for rapid prototyping, not for fabrication of the final product. The resin from which SLA components are fabricated withstands autoclaving, is not toxic to cells (once thoroughly washed), and is very cost effective. It can thus be used for large-scale product manufacturing and may help offset some of the huge scale-up costs that have plagued the tissue engineering industry. For the machinery of such a chamber, first, silicone rubber (Silicones, Inc, NC) are poured into the Petri dish to a height of 5 mm. Blocks (60 ×18 ×18mm) are then placed in the dish and more silicone rubber is poured into the dish to a further height of 6 mm, and allowed to set overnight. The blocks are then removed, leaving a rectangular well. Strips of glass fiber (Millipore, Ireland) are wrapped around the ends of stainless steel rods to act as anchors for the constructs. These rods pass through the holes in the annular ring of the culture dish and connect to the cam followers. It is assembled before steam sterilization. The distance between the two ends of the pull rods is fixed at 36 mm. This is also the length of the collagen constructs.

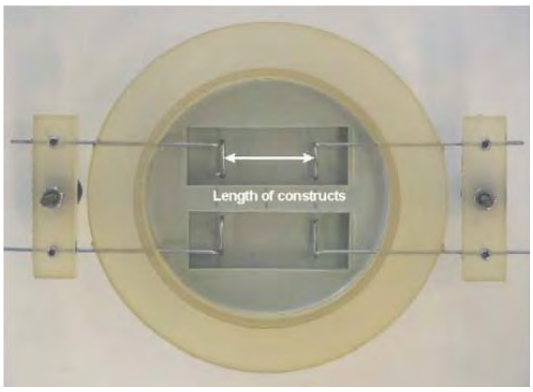


Fig. 63: Top view of culture dish and cam follower (202).

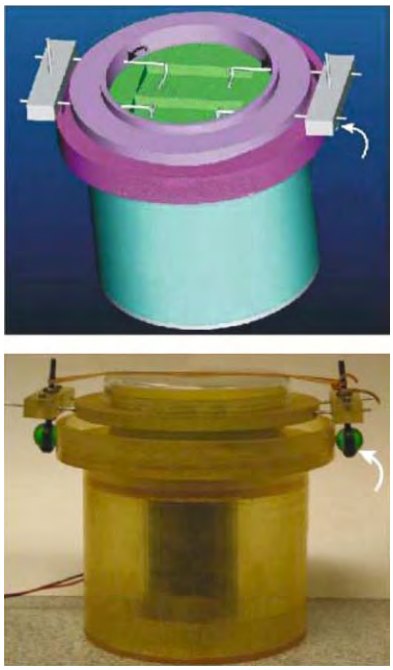


Fig. 64: Top view and the lateral view of the culture dish and the cam follower, assembled prior to sterilization (202).

Chambers with cylindrical geometries have the aim of minimizing flow turbulence, reducing the number of dead angles. Fig. 65 represents other examples of culture systems with cylindrical and spherical chambers. Computational fluid dynamics studies over those models have been conducted by Singh and Hutmacher (203) (204).

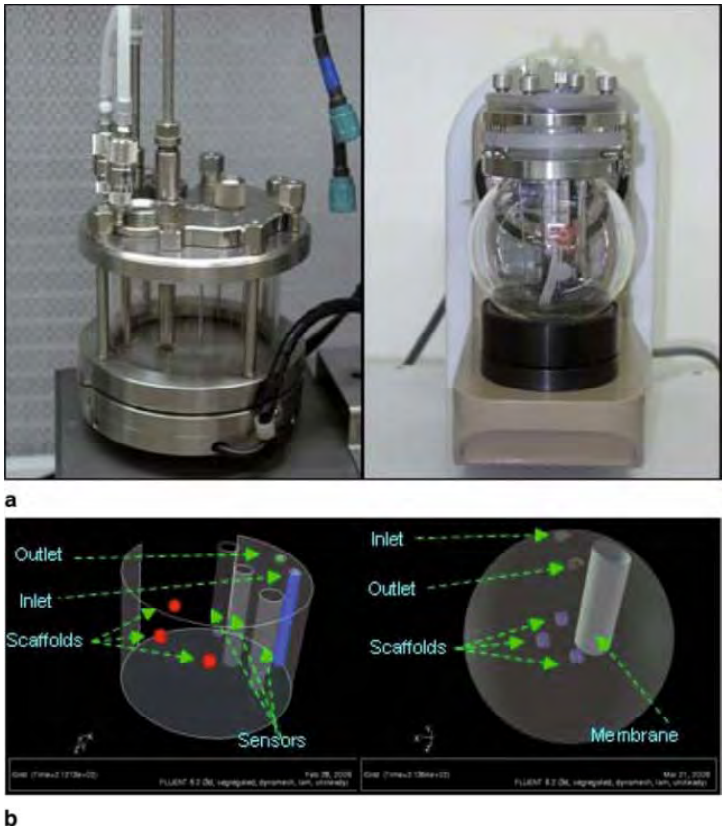


Fig. 65: Cylindrical chamber (left) and new Mk. 3 spherical chamber (right). The complex cylindrical chamber houses multiple probes which are likely to interfere with the flow dynamics of culture media to a greater extent than the spherical vessel and (b) model of cylindrical (left) and spherical vessels (right) (203).

Other researchers, as Ruel et al. (205), introduced cylindrical chambers in their culture system (Fig. 66). The system of Ruel et al. is made of two acrylic blocks into which flow chambers have been machined.

Fig. 66: Bioreactor elements: ventricular pump (1), valve holder (2), compliance chamber (3), variable resistance (4), reservoir (5), bi-leaflet unidirectional valve (6), and flow sensor (7). The compliance chamber (component 3) is located between the valve holder and the variable resistance (component 4) to provide elasticity to an otherwise mostly incompressible system. The role of this location is to simulate aortic and arterial compliance. A custom designed bi-leaflet checkvalve (component 6), represented in detail opens to allow fluid to enter the pump when the membrane moves downward, and closes during systole as the membrane moves upward and flow is produced in the circuit (205).

A flexible membrane (natural rubber, 3 mm thickness) separates the two chambers hermetically. The lower chamber (volume = 206 mL) is filled cyclically with compressed air (driving fluid), forcing the rubber membrane to deflect upward thus forcing the ejection of a certain stroke volume of culture media (circulating fluid). In order to minimize flow turbulence and promote velocity uniformity, the upper chamber presents a specially designed smooth convergent geometry (volume = 266 mL) (Fig. 67).

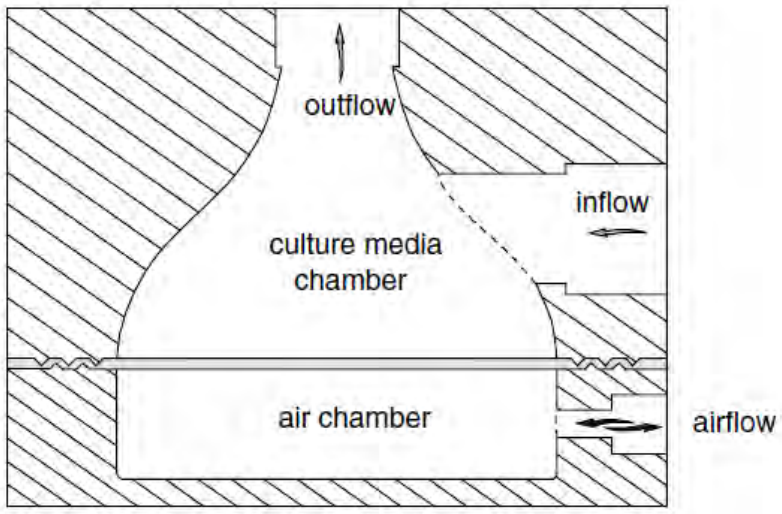


Fig. 67: Schematic representation of chambers and flow flux directions (205).

The chamber is a custom made glass reservoir designed to contain a certain volume of physiological fluid at the bottom, while the upper section is filled with air. The compressibility of air provides elasticity. The

chamber presents a total volume of 1300 mL and is designed to accommodate air volumes up to 900 mL. A static pressure varying from 60 to 100 mmHg can be applied to pressurize the system.

A custom designed bi-leaflet checkvalve (component 6 on Fig. 66, represented in detail in a vertical position on Fig. 68, a to c) opens to allow fluid to enter the pump when the membrane moves downward, and closes during systole as the membrane moves upward and flow is produced in the circuit.

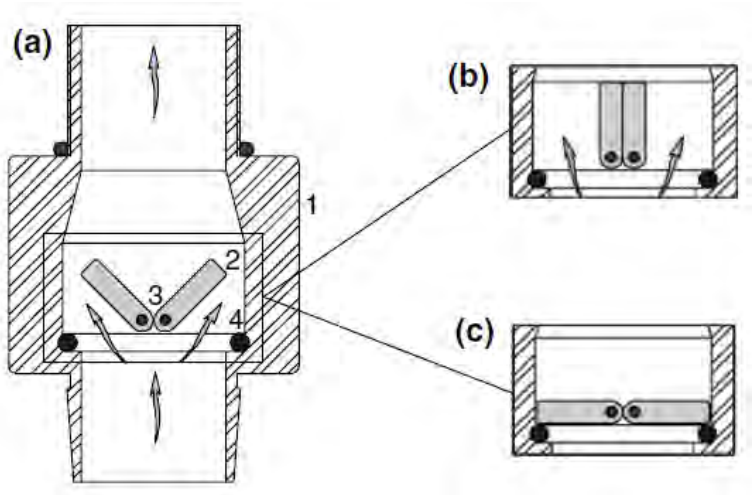


Fig. 68: (a) section view of the specially designed check-valve with leaflets in a transitional movement, 1: check-valve cage, 2: leaflets, 3: leaflet pivots, and 4: silicone o-ring; (b) partial section view with fully opened leaflets; and (c) with closed leaflets (205).

The air is compressed from this initial pressure to a maximum pressure which depends on the initial volume, the stroke volume and the amount of restriction provided by the variable downstream resistance. Depending on the air volume in the chamber, compliance values up to 1 mL $mmHg^{-1}$ may be achieved.

Another model of cylindrical chamber has been developed by Damen et al. (206). The chambers are machined in order to be inserted into a pulsatile bioreactor system. The bioreactor system is entirely made of acrylic plastic for sterilization purposes and consists of three main compartments: air chamber, pressure chamber and perfusion chamber (Fig. 69).

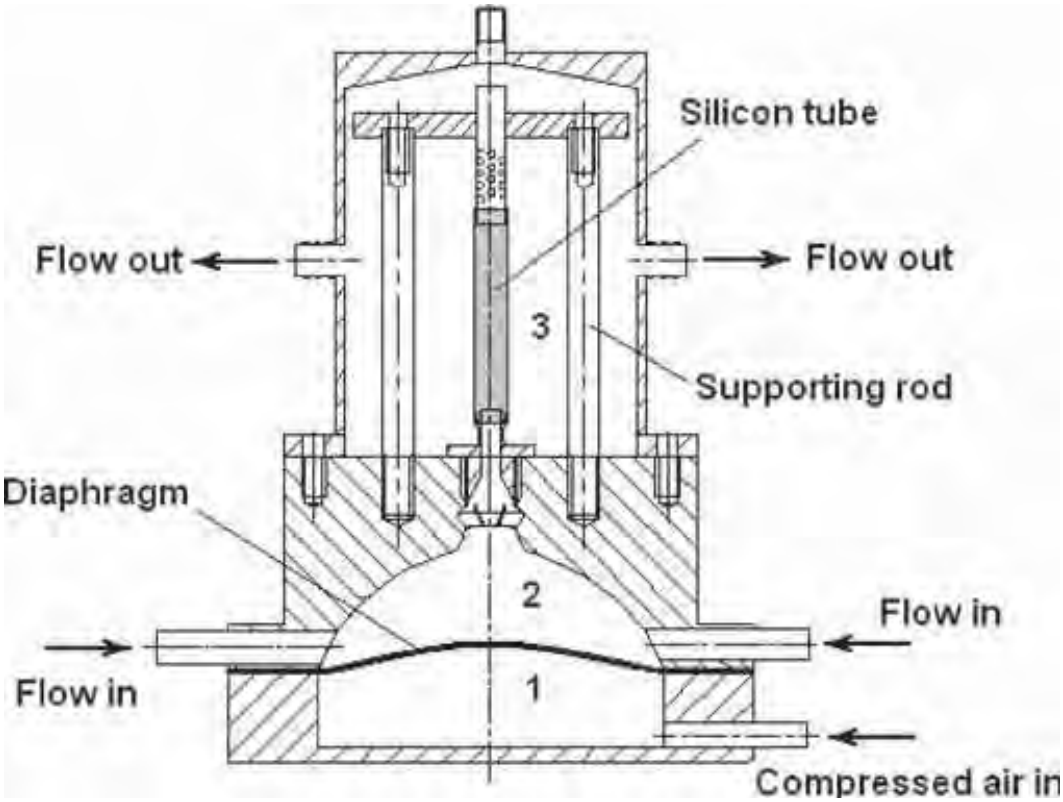


Fig. 69: The air chamber (component 1), that when filled, cause the silicon diaphragm to vibrate, pushing the medium from the pressure chamber (component 2) to the perfusion chamber (component 3), where the cell-seeded scaffold is attached. A silicon tube is used in place of the scaffold in the current setup. Backflow from the perfusion chamber to the pressure chamber is prevented by a one-way bileaflet aortic valve (206).

Air is introduced into the air chamber via a pulsatile airpump which mimics the cardiac cycle (Fig. 70). A silicon rubber diaphragm is attached, akin to a drum skin, between the air chamber and the pressure chamber. The pressure differential results in fibrillation of the diaphragm, causing the diaphragm to simulate heartbeats. The pressure chamber is a 220-ml chamber filled with growth medium such as Dulbecco's modified Eagle medium (DMEM) (Gibco, Invitrogen, Carlsbad, CA, USA), which then flows into the perfusion chamber under the action of the fibrillating membrane. The strategic positioning of the two inlets at tangential positions as well as the spherical shape of the pressure chamber remove the dead angles, ensuring that the growth medium is distributed consistently throughout the bioreactor. Tissues of interest would be grown in the perfusion chamber. Chambers of different shapes and geometries can be incorporated, depending on the type of tissue to be engineered. The chamber consists of a concentric ground glass inlet with a removable stainless steel rod inside, which can be adjusted for different lengths of arteries.

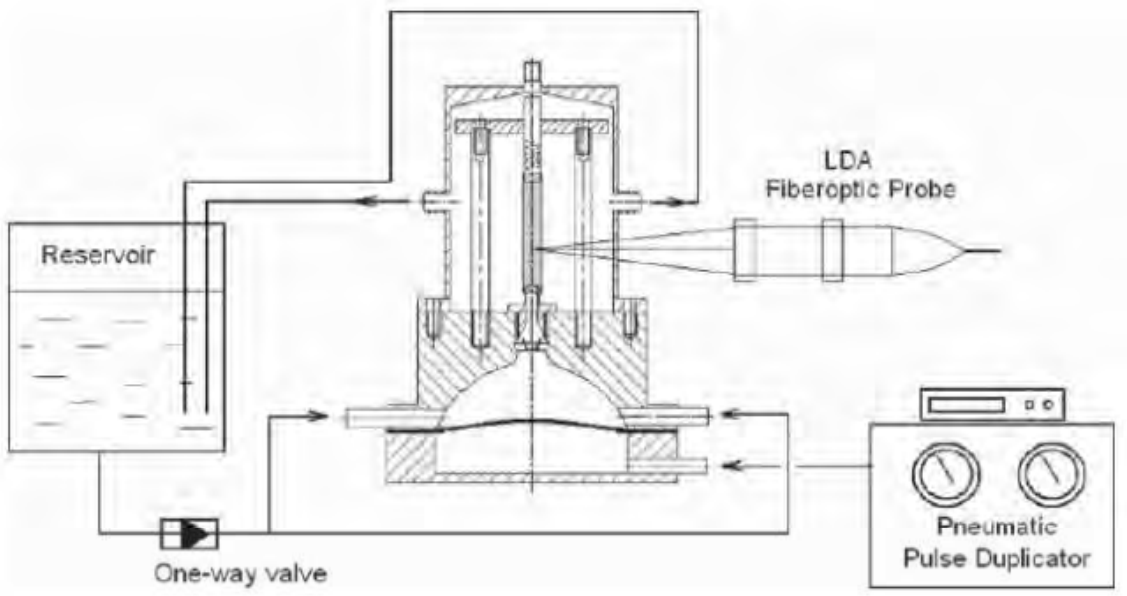


Fig. 70: The outlets are located halfway along the perfusion chamber to enhance the circulation of the growth medium. As the diaphragm returns to equilibrium, the medium progresses from the reservoir in the pressure chamber to the perfusion chamber via a one-way valve that prevents backflow (206).

Some commercially available bioreactor systems also consist of a cylindrical chamber. An example in this sense is the Radnoti® Hi-Tech Heart Chamber (Fig. 71), which allows the cultivation of organs and complex tissues. Its design is optimized for maintaining the organ and permitting specialized instrumentation to be used. The chamber is fully water-jacketed using quick disconnect fittings to permit easy removal from the rest of the system for cleaning. Both the chamber body and lid are independently mounted on support rods for easy positioning. The chamber has a cantered side access port and four ports in the chamber lid for insertion of instrumentation such as balloon catheters, electrodes, fiber-optic cables, cannulae, etc. The chamber lid is secured with a threaded ring, which permits sealing the chamber for reduced heat loss and purging of the chamber interior with gas mixtures. Two of the lid access ports are placed on a locking cam for easy positioning and securing of mounting cannulae, which come in a variety of sizes. The cannulae are fitted into the ports using collars, which allow the cannulae to be rapidly extended or retracted and then locked into place. At the bottom of the chamber is a removable pulley which inserts into the chamber interior and is used for apical force measurements. This pulley can be easily positioned as it is secured with a collar tightened from the outside of the bath.

Fig. 71: Radnoti® Hi-Tech Heart Chamber.

Another commercially available tissue bioreactor is the Mayflower™ tissue bath. It is a horizontal tissue chamber with an integral contraction force measurement. It is completely open at the top and has a small bath volume of 3 - 5 ml. The compact and modular construction provides ideal conditions for investigations on small muscle preparations (urethra, papillary muscle, cavernous body), vascular rings, or tracheal rings.

The basic equipment consists of: acrylate support, tissue chamber, multi-channel roller pump and thermocirculator (Fig. 72). Acrylate support has a movable mounting platform for preload adjustment through a micrometer screw; this platform acts as holder for the force transducer HSE-HA F-30 or F-10. The acrylate support also includes a glass heat exchanger for the pre-heating of the perfusate solution. The tissue chamber is carved out of a rectangular acrylate block and placed on the support stand. This chamber normally includes the tissue holder, the connections for electrical stimulation electrodes, a frit for aeration and a draw-off tube with height adjustment to set the bath volume. The horizontal arrangement of the tissue and the open top provide ideal conditions during preparation and experiment. The tissue bath is available as a complete set-up for 1 up to 8 channels. Two connection sockets for the stimulation electrodes are located on either side of the tissue chamber. Different types of electrodes are available. The solution flows in from a roller pump, the outflow is under suction by the same pump.

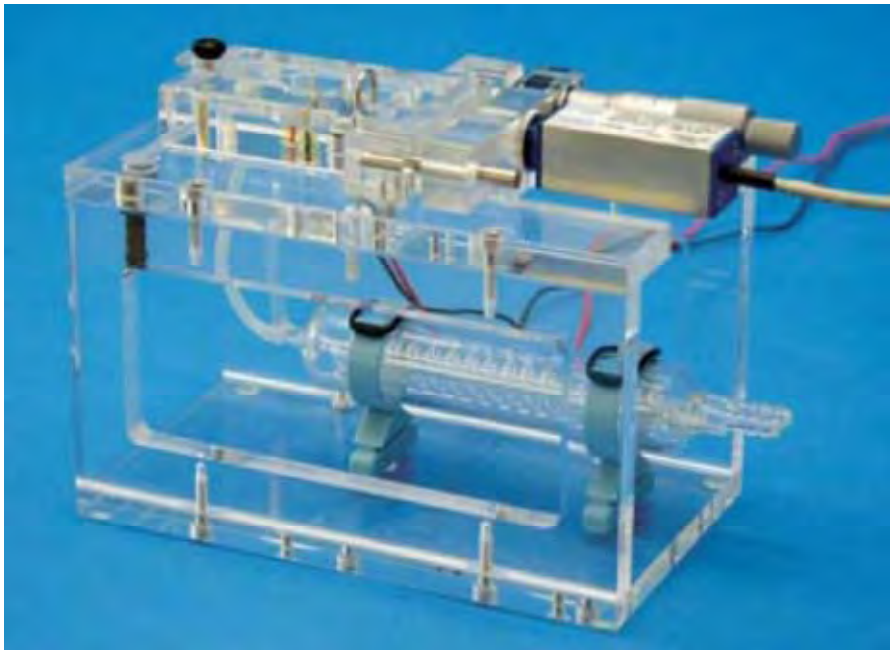


Fig. 72: Mayflower™ tissue bath bioreactor system. The modular concept of this apparatus offers a wide range of different arrangements to meet individual requirements regarding bath geometry, tissue holders and stimulation electrodes.

The features of some of the aforementioned bioreactor chambers are compared in Table 3. Our perspective is that a visual and schematic comparison of the principal design requirements for chambers, seals and latches can support the rational choice and the plan of a new bioreactor system.

Table 3: Comparison among different bioreactor chamber technical features.

Material	Geometry	Properties	Sterilization	Seals	Latches
Polysulfone (197)	Rectangular	Termal and chimical stability, abrasion resistant and optical transparency.	Gas sterilization by ethylene oxide	Neoprene gaskets	Stainless steel screws
Glass (198)	Cylindrical		Autoclave	Silicone-rubber septa	
Polyetheretherketon (PEEK) (199)	Rectangular				
Polytetrafluoroethylene (PTFE) (200)	Cylindrical		Autoclave		
Silicone rubber (201)	Rectangular	No toxic to cells and cost effective	Autoclave	Glass fibers	Stainless steel rods

Glass (205)	Cylindrical		Autoclave	Rubber membranes	
Acrylic plastic (206)	Cylindrical Rectangular (Mayflower)		Gas sterilization by ethylene oxide		Stainless steel rods
(Radnoti Hi-Tech Heart Chamber)	Cylindrical			Threaded rings	

2.2.2. Pumps and fluids

Pumps

In this section, we illustrate the main features of the pump system module of existing bioreactors for tissue engineering. For cell culture, the choice of the pump employed is specific for the bioreactor type and the culture conditions. Some examples are illustrated in the following paragraphs, where a brief analysis of the previously employed pump modules for tissue engineering constructs is developed. In particular, we will address: positive peristaltic pumps, rotating peristaltic pumps, mullti-channel peristaltic pumps, as well as recent alternatives like centrifugal pumps, magnetohydrodynamic pumps, passive gravity head-pumps. We will present a brief explanation of each pump kind previously mentioned, in relation to its particular function in specific bioreactor culture conditions.

Mass perfusion bioreactors often use positive displacement (peristaltic) pumps to circulate the media. We must remember that in a perfusion bioreactor tool, the culture medium has to be continually renewed to supply gas and nutrients to cells and to remove metabolites and catabolites. Thus, the complete perfusion system is required to be composed of an oxygenator, a pump and a medium culture reservoir as shown in Fig. 73. All, or a portion, of the culture medium can be recirculated with or without a supply of fresh medium. In order to obtain physiological values of pH, pO2 and pCO2, the flow media is pumped through long, small-diameter, gas-permeable silicone tubes, which allow a continuous exchange of gases . Obviously, the gas-permeable silicone tubes provide a large surface for gas exchange by diffusion due to the small inner diameter of the tubes (1 mm) and its extended length (1 m). Usually, pumps can produce pressure values of 270 ± 30 mmHg , with variable stroke volumes of 0–10 ml per stroke and the bioreactor can be operated at a range of pulse rates from 60 to 165 beats per min (207).

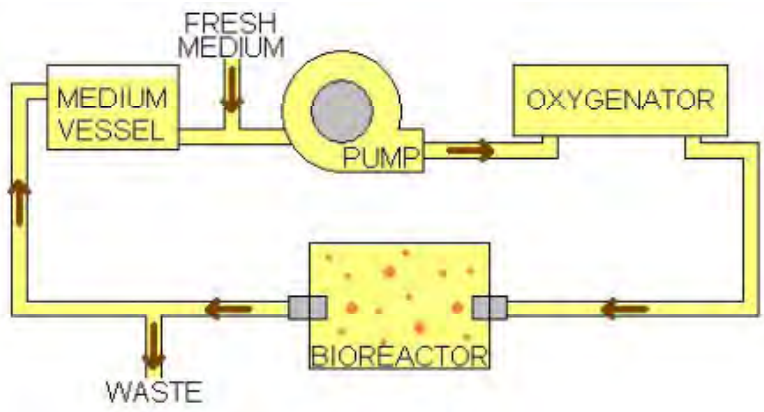


Fig. 73: Perfusion system composed by an oxygenator, a pump and a medium culture reservoir (207).

Developing tissue have to be supplied with a continuous or pulsating flow of fresh, oxygenated culture medium in order to avoid unstirred layers of fluid and to achieve optimal differentiation. This is best accomplished using a slowly rotating peristaltic pump able to deliver adjustable pump rates of 0.1 to 5 ml per hour. An example in this sense is represented by the case of cardiac patches, where the pump provides an adjustable flow that can be continuous or pulsatile with a frequency range of 50 to 200 beats/minute to cover the range from foetal to adult cardiac frequencies (208). The culture medium is generally transported through bottle screw caps and tubing that consist of various materials. Typical materials along the medium transport path are a glass tube and large diameter silicon tubing within the storage bottle, polysulfonate bottle caps, connectors made from polypropylene and small diameter silicon tubing through the pump and towards the culture container. The culture medium is saturated with oxygen during its passage through silicon tubing to guarantee optimal supply for the developing tissue (209).

In the bioreactor represented in Fig. 74, the peristaltic pump maintains a flow of fresh culture medium from the storage bottle through the gas exchanger. The medium continues through the gas expansion module where remaining gas bubbles are eliminated and on to the perfusion culture container that holds the tissue construct. Used medium that leaves the culture container is not recycled but is collected in separate waste bottles (210).

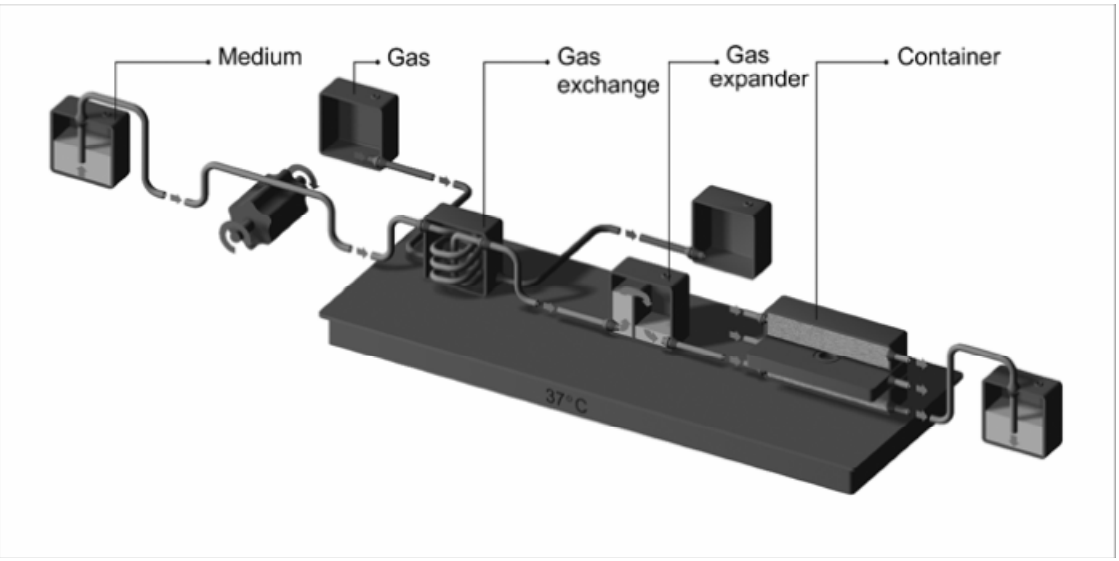


Fig. 74: The working line consists of: a storage bottle for culture medium, a peristaltic pump, a gas exchanger, a gas expander to trap gas bubbles, a culture container and a bottle to collect the waste medium (210).

Gas exchanger, gas expansion module and culture container are placed on the surface of a heating unit that maintains a constant ambient temperature. All modules and heating plate are covered with a protective Plexiglas lid. This setup is self-contained to maintain sterility and allows culture of tissue constructs under reproducible conditions for extended periods of time.

The scheme in Fig. 75 gives an illustration of the already mentioned flow-chamber bioreactor system proposed by Nagel-Heyer et al. (199), where a peristaltic pump (Medorex™) allows the fluid to circulate from a conical conditioning vessel thought the cultivation chamber.

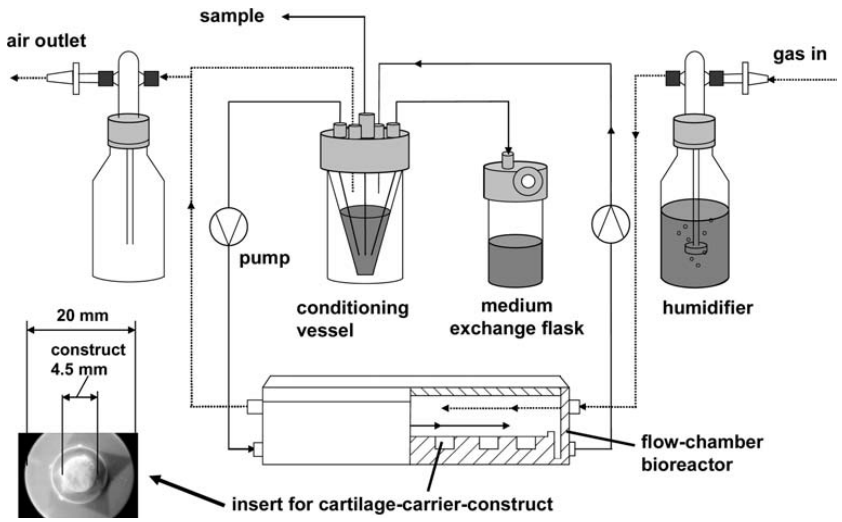


Fig. 75: Flow-chamber bioreactor system, consisting of: flow chamber, conditioning vessel, humidifier, exhaust flask, medium exchange, insets for cartilagecarrier-constructs (199).

In their perfusion culture of chondrocytes, Strehl et al. (211) used a IPC-N 8 peristaltic pump (Ismatec,Germ any) activated at a rate of 1 ml/h (Fig. 76). In the culture of osteochondral grafts carried out by Wendt et al. (212), an oscillating perfusion was induced from the pumping of the headspace from one vertical column to the other. This pumping is aimed at seeding cells uniformly throughout the porous scaffold. Following the cell seeding phase, a set of valves are switched and the flow is diverted to an alternative path for prolonged culture.

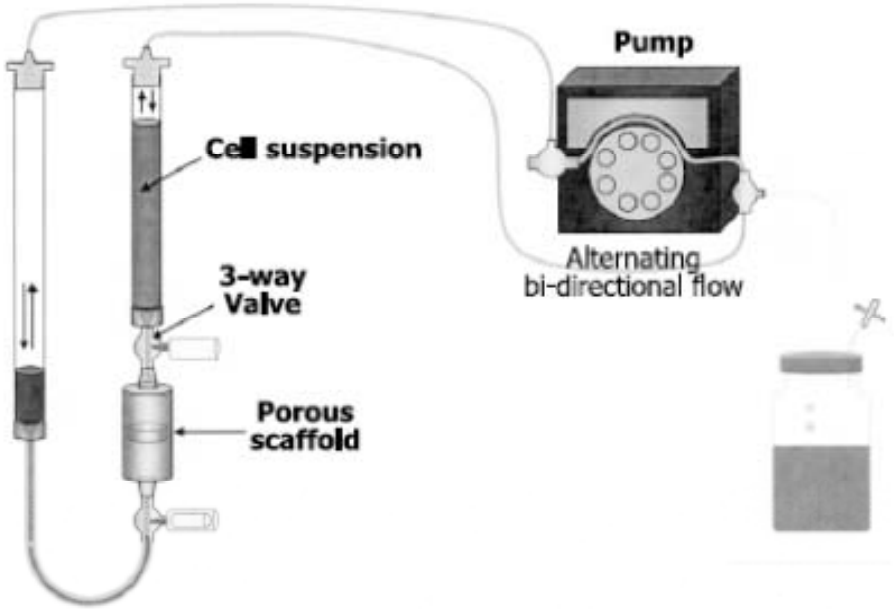


Fig. 76: Schematic diagram of the pumping system in a perfusion culture of chondrocytes (211).

Another type of pump used in tissue engineering cultures is the mullti-channel peristaltic pump, useful in case of several culture lines running in parallel. An example in this sense is represented by the system for human mesenchymal stem cells of Zhao et al. (213). The system consists of four perfusion chambers, a media container, a fresh media container, and a peristaltic pump with eight flow channels. The whole system except the pump is placed in a 378C incubator to maintain a constant operating temperature Fig. 77.

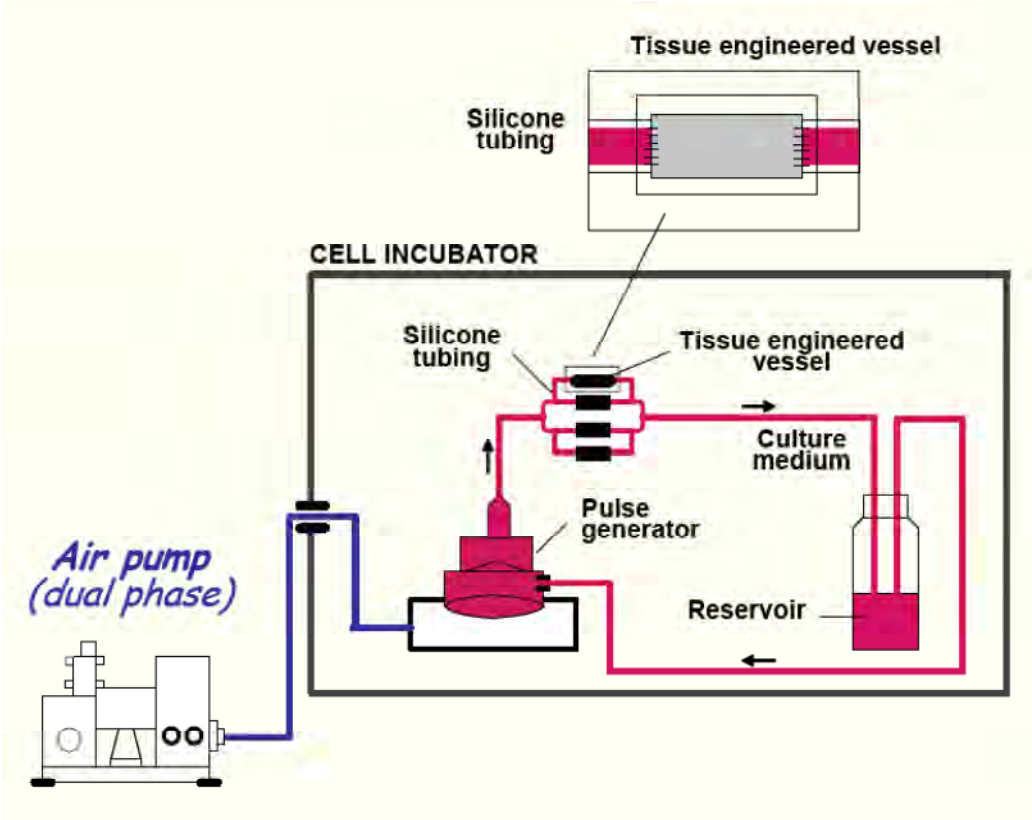


Fig. 77: Bioreactor system developed by Zhao et al. The dual phase peristaltic pump has eight flow channels (213).

There are two sampling ports at the inlet and the outlet of each chamber that provide media sampling for analyzing biological parameters and oxygen tension. The pH and oxygen levels of the media are controlled by the composition of a gas mixture pumped into the gas phase of the media container through a 0.2 mm sterile filter. The two inlets and two outlets in each chamber are connected to independent inlets from the computerized precise peristaltic pump (Cole-Parmer, Vernon Hills, IL) that has eight independent channels. Media circulation is driven by the pump and the flow rate is controlled by adjusting the pump head. During operation, media is drawn from the media reservoir, pumped through the compartments, merged at the outlets of the chambers, and returned back to the media reservoir. There are two sampling ports at the inlet and outlet of each chamber that provide media sampling for analyzing biological parameters and oxygen tension (Fig. 78).

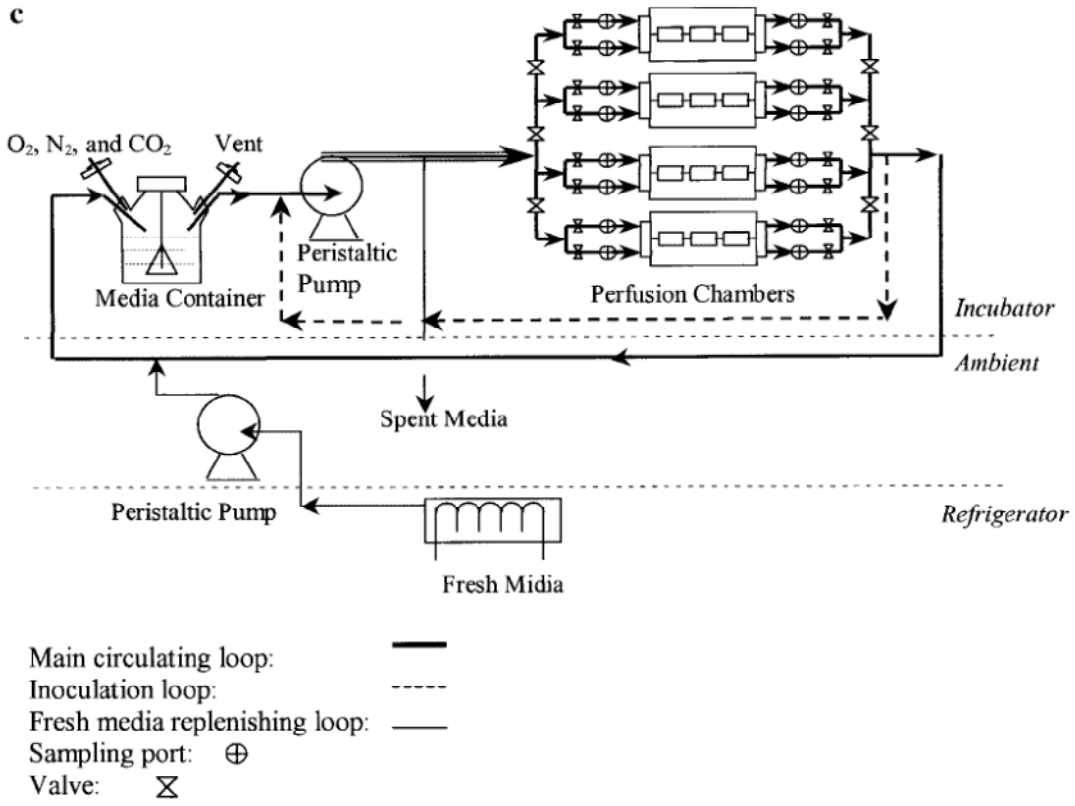


Fig. 78: Bioreactor system developed by Zhao et al. The multichanel peristaltic pump has eight flow channels.

The perfusion system of Bancroft et al. (214) makes also use of a multichannel peristaltic pump. In this system, the pump driving the flow is a six-channel peristaltic pump (Cole Parmer, Fig. 79). This pump gives accurate and consistent flow rates from 0.1 to 10 mL/min with the tubing size used in our system (Cole Parmer L/S 16). Because of the peristaltic nature of the pump and the relatively lower mechanical durability of silicone tubing, neoprene tubing (Pharmed tubing; Cole Parmer), a more rigid but non-gas-permeable tubing is used for a short segment of the circuit within the pump.

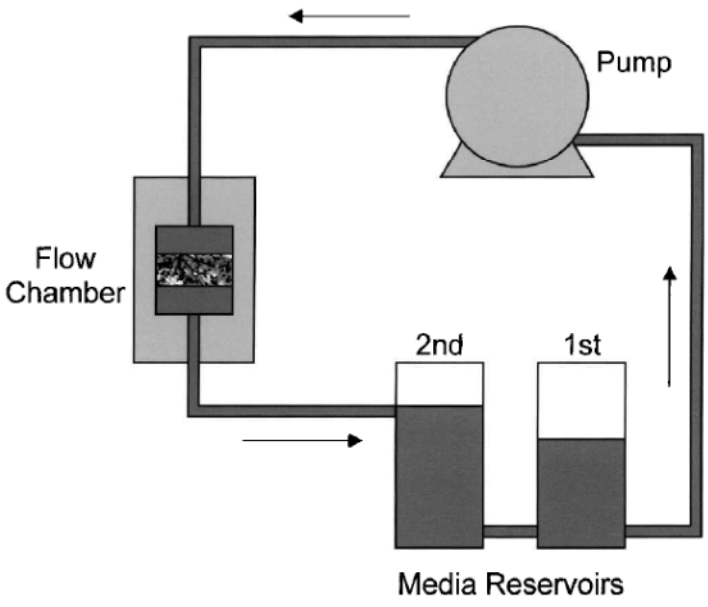


Fig. 79: Diagram of the perfusion pumping system developed by Bancroft et al. (214).

Unfortunately, mechanical friction and compression of cell culture media within silicon tubes often results in precipitation, aggregation, and loss of proteins within the serum. Many of the proteins lost in this manner are the chemical factors within the serum that promote muscle tissue growth and development. Other types of perfusion pumping systems, especially those that involve rapidly moving turbines or valve elements or sliding contact mechanism seals are also thought to cause damage to both whole blood and cell culture media (215). Therefore, the development of more appropriate methods of perfusion pumping is required.

Hong et al. (216) have employed a perfusion bioreactor system for the in vitro development of vascular grafts, based on a centrifugal pump. The system deliveres physiologic, arterial, pulsatile intraluminal pressure (120/80 mmHg) at minimal flow (w10 mL/min). The Biomedicus centrifugal pump connected via Tygon tubing to a tissue testing chamber produces sinusoidal pulsatile pressure and flow consistent with physiologic values. An additional flow loop consisting of a roller pump (Masterflex, Cole-Parmer, Vernon Hills, IL) and a heat exchanger placed into a water bath (Fisher Scientific) recirculates warm saline into the chamber to maintain a temperature of 37°C during testing.

Other possible alternatives include magnetohydrodynamic pumping (217), and passive gravity head-pumps (218). In the passive pumping method, the surface energy present in a small drop of liquid is used to pump the liquid through a microchannel. Fluid can be pumped through a microchannel by using the surface tension in a drop of liquid and two or more ports on the microchannel—a reservoir port and a pumping port. A large drop (e.g., 100 μL) is placed over the reservoir port of a fluidfilled microchannel. The radius of this drop is large enough to cause the pressure at this port to essentially be zero. A much smaller drop (e.g., 0.5–5 μL) is placed on the pumping port, and because of its smaller radius, a larger pressure exists at this end (Fig. 80). The flow rate is determined by the volume of the drop present on the pumping port of the microchannel. A flow rate of 1.25μL s^{-1} is demonstrated using 0.5 μL drops of water. Two other fluid manipulations are demonstrated using the passive pumping method: pumping liquid to a higher gravitational potential energy and creating a plug within a microchannel.

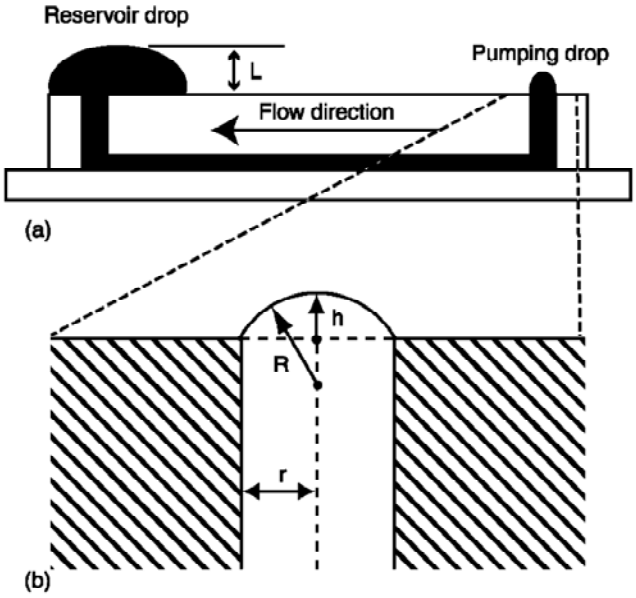


Fig. 80: (a) Side view of a microchannel. A reservoir port with a large drop and pumping port with a smaller drop are required for fluid flow. (b) A drop of volume V will form a spherical cap of radius R on a port of radius r. The cap will rise above the surface of the device a distance h. If the drop volume is less than that for a hemisphere of radius r, then the drop radius, R, will be larger than h.

Common criticities in the pumping system module

Typically, the medium is drawn up from the bottom of a storage bottle through tubing and passes through the bottle cap before reaching the continuing tubing. In this scenario, the pump's suction pressure has to overcome the difference in elevation within the storage bottle as well as the capillary force resulting from thin tubing. This usually does not cause problems at high pump rates. At low pump rates, however, medium is insufficiently aspirated from the bottle and the amount of air bubbles increases . These microscopic gas bubbles are transported along with the culture medium, increase in size and eventually form an embolus that massively impedes medium flow. Gas bubbles can also accumulate in the culture container where they lead to a regional shortage of medium supply. Air bubbles cause additional problems in both storage and waste bottle where growing air accumulations cause erratic breaks in the fluid continuum. In small diameter tubing this causes massive pressure changes (219). In a gradient culture setup where two media have to be transported at exactly the same speed and pressure such effects can lead to pressure differences which in turn can destroy the growing tissue. Consequently, to address the problem of gas bubble formation the medium transport path has been redesigned from some authors using only one material and tubing of a small and constant diameter. New bottle caps maintain sterility within the storage bottles (Fig. 81).

Fig. 81: Bottle cup and small diameter tubing.

One opening in the cap is designed for a continuous piece of tubing reaching from the bottom of the storage bottle to the inlet of the culture container thus avoiding material transitions along the fluid path. This considerably reduces bubble formation. Another opening holds a sterile filter allowing sterile air to enter the storage bottle as medium is drawn from it. This opening can also be used as an inlet for medium or gases.

Damage to the perfusate, especially the dissolved proteins, is thought to result at the liquid–gas interface present in most perfusion systems. Another possible solution is the use of compliant reservoirs which can expand and contract to accommodate changes in perfusate volume, as well as an overall zero head space bioreactor design (220) (221), to eliminate all liquid–gas interfaces by interposing semipermeable membranes where this interface might normally occur.

Circulation systems and general composition of fluids and gases

In this section, we show the functions and the principal characteristics of fluid circulation in a bioreactor system for tissue engineering. Fluid circulation provides the distribution of nutrients and the removal of metabolites and catabolites. An example of this concept is represented by the scheme of the hollow fiber bioreactor for bone tissue engineering. In this type of bioreactor the distance from the nutrient supply for every cell can be kept to a minimum. Cells can be seeded and cultured onto the outside of the fibers to

mimic osteon formation with nutrient supply being provided through the lumen of the fibers. In the hollow fiber bioreactor not only will the bulk of the fluid pass through the channels and not reach the extremities of the packing but also the immediate cells will experience increased shear. The system is schematized in Fig. 82.

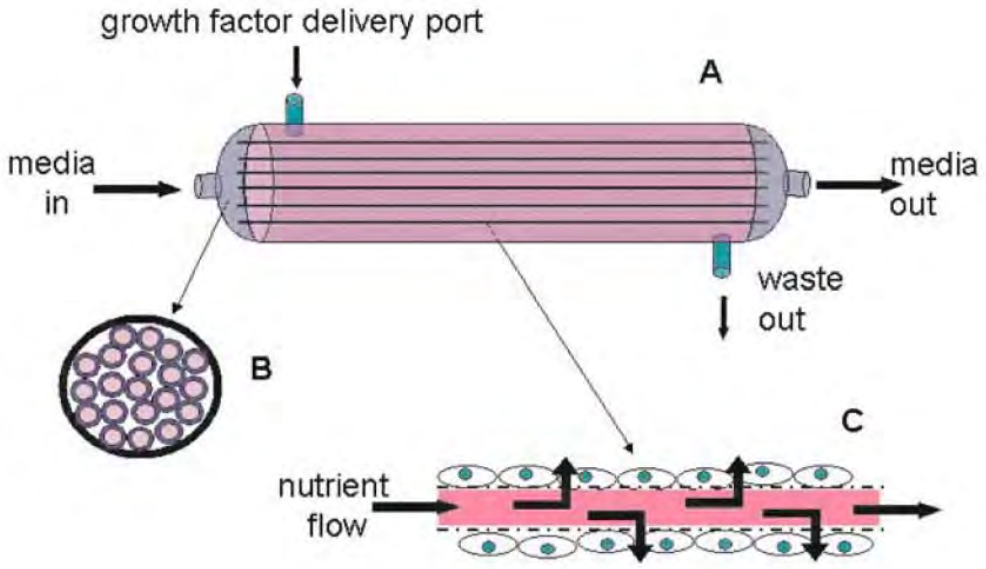


Fig. 82: Hollow fiber bioreactor system.

Notice that, in addition to nutrients transport and products removal, fluid flow is also needed in order to subject cells at precise biomechanical stimulus. Both functions are, in fact, essential for the survival and the systematic development of the cellular construct.

In culture of cardiac patches, the provision of nutrients and gases to nascent cell construct is probably the limiting factor for the creation and maintenance of a multi-layer vascularized tissue. Mass transfer within the entire cell construct involves the transport of nutrients and gases from the place that they are supplied to the site of the cells. Generally, two processes govern mass transfer of nutrients to cells: convective mass transfer or the transport of molecules in the bulk motion of the media flow; and diffusion, the movement of species down a concentration gradient. As reported in current literature (222), the design of bioreactors must be such that the fluid flow system provides concentrations of nutrients, oxygen and growth factors homogeneous at 100 µm scale. Indeed, a tissue section of this size represents the organ functional subunit, with a variable number of cells, according to the tissue type. For example, in cartilage there is approximately one cell per 100 μm^3 (222), whereas a tissue section contains generally a mixed cell population of about 500 – 1000 cells. In tissue engineering, convective mass transfer get the nutrients to the vicinity of the cells. From this point it will be molecular diffusion that governs transfer through the cell layers. The ratio between convective transport and diffusive transport is a key consideration (Peclet number). At the cell surface the nutrients will be taken up and here there is a balance between the rate at which the nutrients are taken up and the rate at which they are supplied by diffusion (Damkohler number). When this ratio is large, nutrients are consumed more rapidly than they can diffuse to the surface, and the surface concentration will fall. When it is small, the uptake rate is slow and diffusion maintains the nutrient concentrations as nearly constant. Another mass transfer consideration is the choice of a suitable media flowrate to ensure there is continuous supply of fresh nutrient and removal of toxins. We must consider the balance between the residence time of the media in the reactor, the resulting shear rate and its effect on the nascent tissue, and specific nutrient demand requirements. Overall, in vivo rates of oxygen uptake

of 25 – 250 µmol O_2/cm^3/h and perfusion rates of 0.07 mL/cm^3/min, based on an average cellularity of 500 million cells per cm^3, may be good starting points (222). Tissues have very individual oxygen requirements (209). For that reason it is important to regulate the respiratory gas content. A popular method for medium oxygenation is to blow a pressurized gas mixture into the storage bottle. The disadvantage of this method is the formation of gas bubbles in the transported medium. These bubbles accumulate along the medium transport path and cause pressure differences within the system. This method can also introduce infections caused by contaminated gases. Thus, the technical dilemma is to obtain maximum oxygen saturation while avoiding gas bubble formation. One of the solutions is a newly developed gas exchanger module housing a long thinwalled silicon tube for medium to pass through Fig. 83. The tubing is wound up into a spiral inside the exchanger module which features a gas inlet and outlet. The tubing is highly gas-permeable and guarantees optimal diffusion of gases between culture medium and surrounding atmosphere. The desired gas atmosphere within the exchanger is maintained by a constant flow of a specific gas-mixture through the module.

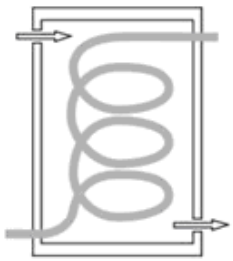


Fig. 83: Gas exchanger module housing a long thinwalled silicon tube. The tubing is wound up into a spiral inside

This way the content of oxygen, carbogen or any other gas can be increased or decreased in the medium by diffusion making it possible to adjust the gas partial pressures within the medium under absolutely sterile conditions. By maintaining a defined carbon dioxide concentration in the medium this method can also be employed to control medium pH via the bicarbonate buffer.

In perfusion culture applications, culture medium with high oxygen content has to be transported while the formation of gas bubbles has to be avoided. Using advanced oxygenation techniques the occurrence of harmful bubbles can be greatly reduced but nevertheless some bubbles will remain in the medium. Consequently, it has been developed an additional gas expansion module that removes remaining gas bubbles from the culture medium. Medium is pumped into a specially designed container, where it rises within a small reservoir. Here the medium can expand and equilibrate before it drops down a barrier (Fig. 84).

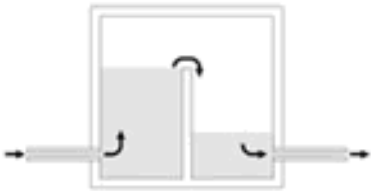


Fig. 84: Gas expansion module

During this process gas bubbles are separated from the medium and collected at the top of the container so that the medium which leaves the container is gas-saturated but bubble free. The gas expansion module itself can be ventilated through a port at the top. This ventilation port can be coupled with the port on a parallel gas expansion module. This way the individual channels of a gradient culture setup can be bridged to obtain identical pressures at the luminal and basal line. Perfusion culture setups are self-contained and can be used on a laboratory table.

In Fig. 85, we show an example of perfusion bioreactor. A peristaltic pump transports the media (1 ml/h) to a gas expander module and then to a gradient container.

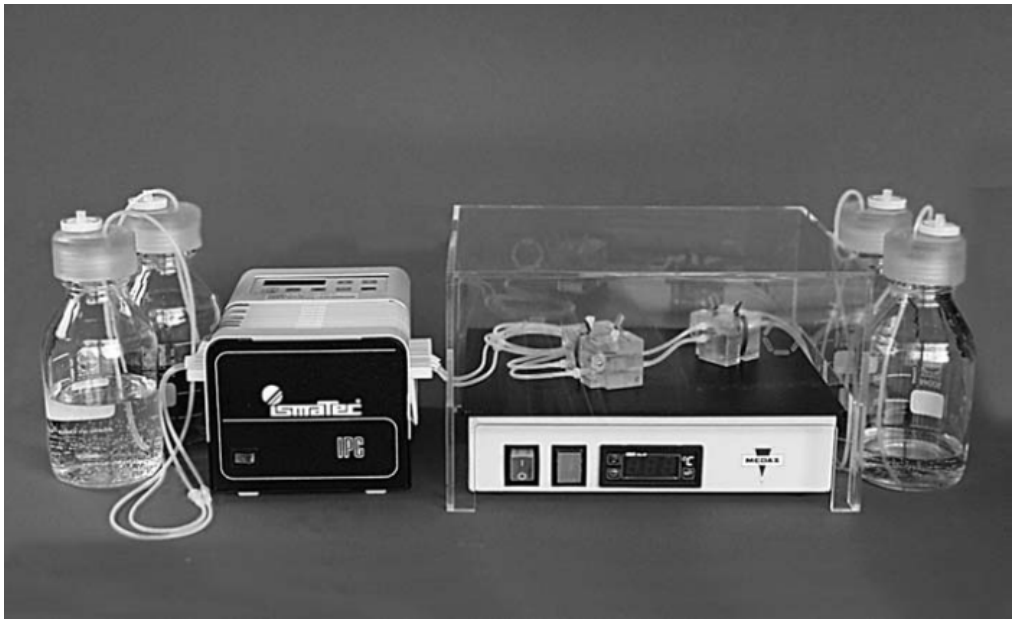


Fig. 85: Perfusion bioreactor system. The waste medium is collected in the bottles on the right side.

There is evidence that mechanical input, such as hydrostatic pressure and fluid flow induces shear during in vitro culture, allowing tissues that have characteristics more like in vivo structures. At its most simple, fluid flow affects cell shape and cell function (195). Since cellular morphology can be affected by the environment, some reactors have incorporated mechanical shear forces to replicate in vivo stresses. Pulsed bioreactors with adjustable stroke volumes and rates have been used to culture cardiac patches made from PGA or collagen (223). Carver and Heath (224) have used a perfusion chamber with a PGA matrix, but also incorporated an intermittent compression cycle using hydrostatic pressure (5 seconds on, 15 s off, for a 20 min period every 4 h). This continued for cultures of 5 weeks and appeared to increase cartilage synthesis. Changes in mechanical load may also regulate ECM gene expression by fibroblasts. Stretched fibroblast matrix upregulated ECM proteins typical to tendons and ligaments (tenascin-C, collagen XII) (225). Specific reactors are being developed to induce stimuli as cultures progress (226).

An example is represented by the aforementioned T-CUP bioreactor (200). T-CUP bioreactor employs the principle of alternating perfusion in order to distribute cells within scaffolds and maintain nutrient supply. The system is represented in Fig. 86.

Fig. 86: T-CUP bioreactor system (200).

A peculiar feature of the system is the fact that perfusion is generated by the motion of scaffolds through the fluid (i.e., a cell suspension or culture medium), with the only possible path for the fluid being through the scaffold pores.

On operation of the stepper motor, the upper reactor cup is driven along its vertical axis in alternating directions. Consequently, scaffolds located in the scaffold housing move through the fluid (i.e., cell suspension or culture medium) in the lower cup, which is forced to pass through the pores of the scaffolds. In particular, on the downward stroke compression pushes medium through the pores, and on the upward stroke expansion of the chamber reverses the flow direction. Gas exchange occurs as a consequence of this cyclic compression and expansion via a port on the dual-upper cup fitted with a 0.2-mm sterile PTFE filter (Acro37, PALL). On the up stroke the direction of perfusion is reversed.

Culture medium composition

It is known that culture conditions influence gene expression and, hence, probably many properties of the cells. Optimization and standardization of culture methods is needed for research as well as for clinical purposes. Hence, a defined culture system with known composition of culture medium is desirable to minimize the variability that affects the reproducibility of research results. Standardized and optimized culture systems are needed, and the normality of the cells has to be followed up regularly. In the following, we illustrate recent culture medium composition for human embryonic stem cells (hESC) culture (227) and for cultured adult cardiac myocytes (228).

Cullture medium for hESC culture

Traditionally, hESC culture medium has contained FBS (229). However, in recent years it has, by most of the teams, been replaced by alternatives.

Richards et al. (230) reported the use of human serum instead of FBS in hESC culture medium. The problem is that FBS as well as human serum are complex mixtures containing unknown compounds, and serum batches vary in capability of maintaining hESC at an undifferentiated stage. Serum may also contain factors inducing hESC differentiation. To avoid these problems, some researchers (231) have optimized serum-free

culture conditions for hESC lines using a basic fibroblast growth factors (bFGF) and a more defined serum replacement (KnockOut Serum Replacement, Invitrogen), which still contains animal proteins, which are not fully defined. During optimization of hESC culture conditions by replacing FBS with serum replacement (SR) in hESC medium, Skottman et al. (227) made the interesting observation that hESC were proliferating faster in SR-containing medium than they did in FBS-containing medium (232). Although the stem cells characteristics of these cells (the expression of many known hESC markers) and their differentiation capacity in embryoid bodies were similar, surprisingly, over 100 genes were found to be significantly differentially expressed when hESC cultured in serum-containing medium were compared with those cultured in SR medium. As a conclusion, Skottman et al. (227) suggested that such changes may have fundamental importance for hESC.

Cullture medium for adult cardiac myocyte culture

The basic, ''non-supplemented'' medium routinely used to culture adult heart cells is bicarbonate buffered Medium 199 (233). Medium 199 contains all amino acids (except glutamine), vitamins and the following ionic constituents (in mmol l-1): $CaCl_2$, 1.8; NaCl, 116; Na acetate, 0.6; NaHPO , 1; KCl, 5.3; MgSO , 0.8. Common additional supplements are (in mmol-1); creatine, 5; taurine, 5; L-carnitine, 2; pyruvate, 2.5; and insulin 10-7 M (233). To prevent bacterial infection all media contain 50 I.U. penicillin and 50 mg ml-1 streptomycin. The cells are maintained under sterile conditions in an incubator in a 5% CO –95% air atmosphere at 37°C.

2.2.3. Sensors and Control

Sensing in tissue culture bioreactors represents a major method to clarify still unknown aspects of the cellular response in dynamic culture conditions, as well as a step forward towards the automation and in-process control of tissue manufacturing processes. Monitoring the partial pressure of O_2 and CO_2 in the culture medium, or detecting the concentrations of glucose and lactate, for instance, allows quantitative evaluation of the metabolic behaviour of cultured cells, thus supporting/substituting subjective, qualitative conclusions traditionally derived by simply observing the colour of the medium.

The noteworthy parameters which should be adequately monitored and controlled during in vitro organogenesis can be classified in two main categories, namely the milieu parameters and the construct parameters. Milieu parameters are then physical (e.g., temperature, pressure, flow rate), chemical (e.g., pH, dissolved O2 and CO2, chemical contaminants, concentration of significant metabolites/catabolites such as glucose, lactate or secreted proteins) and biological (e.g., sterility). Similarly, the construct parameters can be different in nature: physical (e.g., stiffness, strength, and permeability), chemical (e.g., composition of the scaffold and of the developing extracellular matrix) and biological (e.g., cell number and proliferation rate, concentration of intracellular proteins, cell viability).

Monitoring of the Milieu

Beside the general requirements that sensors need to meet in the common practice (i.e., accuracy, sensitivity, specificity), probes employed in cell and tissue culture are required to fulfil peculiar specifications. In particular, they might need to be rather small in size compared to many commercially available sensors, have a lifetime of several weeks, unless they are sufficiently low cost to be disposed of and replaced during culture, and must ensure stable response over time, since repeated calibration might be difficult to carry out due to accessibility of the sensors during culture. Sensors for milieu monitoring can be generally classified in different categories, according to the position of the sensing probe relative to the culture chamber of the reactor: invasive, indirect and non-invasive sensing (Fig. 87 and Fig. 88).

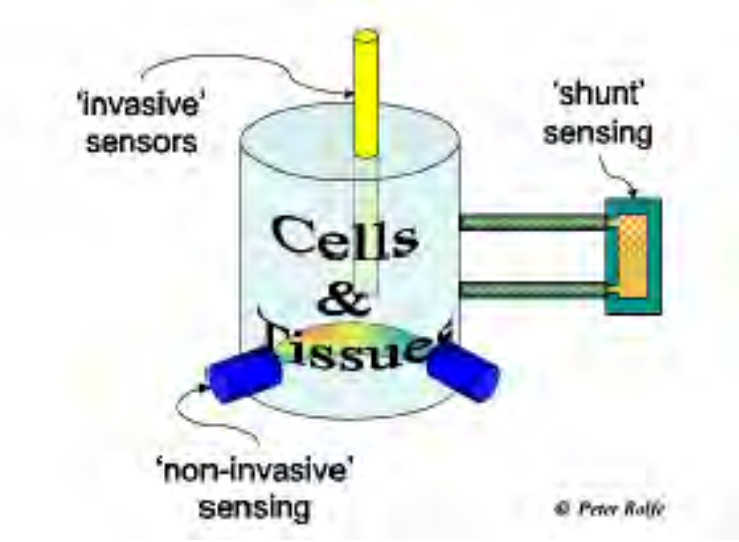
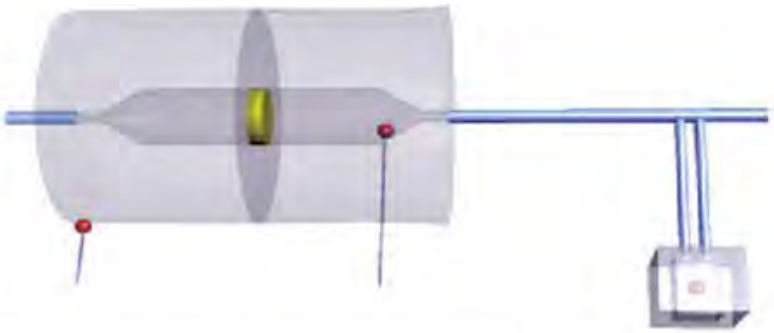


Fig. 87: Schematic representation of sensing systems for cells and tissues grown in a cylindrical bioreactor: invasive, non-invasive and indirect ("shunt") sensing.



● = sensing probe	NONINVASIVE SENSING (non-contact)	INVASIVE SENSING (embedded)	INDIRECT SENSING (offline, shunt)
ADVANTAGES	• no need for sterility • ease of calibration	• high specificity • high sensitivity	• high specificity • high sensitivity
DRAWBACKS	• need for transparency • low specificity • low sensitivity	• need for sterility • difficult calibration	• lag time → artifacts • impaired feedback

Fig. 88: The three main modalities to monitor the milieu in bioreactors. 1. " Invasive sensing " implies that the sensor probe is placed directly inside the culture chamber of the bioreactor, either immersed in the culture fluid or in direct contact with the engineered construct. 2. " Noninvasive sensing " involves sensors that do not come in contact with the interior of the culture chamber, but are capable of measuring via interrogation through the bioreactor wall. 3. " Indirect sensing " is performed directly on the culture media, but via sampling means, either by offline analysis or shunt sensing.

Invasive sensing

Invasive (embedded) are those sensors whose probes are placed directly inside the culture chamber of the bioreactor, either immersed in the culture fluid or in direct contact with the engineered construct. Clearly, invasive sensors must be sterile and therefore either disposable or capable of withstanding repeated

sterilization protocols. Integrating these sensors within a bioreactor for clinical applications may have limitations considering the potential high cost of singleuse disposable sensors. Clear advantages of invasive sensing are the high precision and accuracy achieved with the measurement. The most common invasive sensors currently in use are based on optical and electrochemical principles: fiber optic fluorescence-based sensors, for example, have been manufactured for the measurement of dissolved oxygen and of pH, while typical electrochemical sensors are membranes functionalized with appropriate enzymes for the detection of glucose or urea. Fig. 89 shows an electrochemical sensor that may be used for invasive bioreactor measurement. It is constructed using a 0.8 mm diameter polymer supporting catheter. The diffusion membrane is polymeric and, for example, may be high- or low-density polyethylene, polyetherurethane or plasticized PVC. The membrane thickness may also be adjusted to vary response time (from typically 3 s to 60 s) and current sensitivity. When used for PO2 measurement a Pt microcathode (15 µm) is used with an electrolyte buffered at 7.40 and fixed polarizing potential of -650 mV.

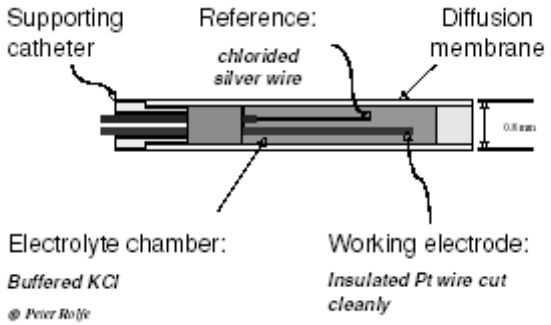


Fig. 89: Electrochemical sensor for invasive bioreactor measurement

Microneedle electrochemical sensors have been developed for direct insertion into cell aggregates or tissue constructs. Enzyme biosensors, for example, for glucose or urea measurement, have been constructed by solvent-casting polyetherurethane membranes over an appropriate enzyme (e.g. glucose oxidase) which has been immobilized, for example, using cellulose tri-acetate. With such glucose enzyme sensors the working electrode is polarized at +450 mV to oxidize the H_2O_2 produced from the catalysed reaction of glucose and oxygen. Invasive fibre optic sensors have also been fabricated. Plain fibre sensors have been used to transport interrogating light to and from the culture chamber to allow measurements of scattering and spectral absorption. Scattering measurement within the culture fluid is being investigated for its potential to provide a gross indication of cell and other particulate mass circulating within the culture fluid. However, a more important aspect of culture fluid analysis is for spectrophotometric analysis. The plain fibre sensor is less suitable in this application than an insertion probe in which there is a fixed geometry analysis chamber, as shown in Fig. 90. The plain fibre probe and the two immersion probes shown in the figure hereafter may be connected to instrumentation for classical ultraviolet/visible (UV–VIS) or visible/near infrared (VIS–NIR) spectrophotometry, including FTIR. This approach requires model building, for example, with data sets generated from the particular culture process being studied. There is a special interest in the present study to monitor glucose as a potential control variable in a closed-loop bioreactor. In addition to the use for full spectral analysis these probes, with appropriate fibres, are also suitable for fluorescence measurements, either by exploiting the natural fluorescence

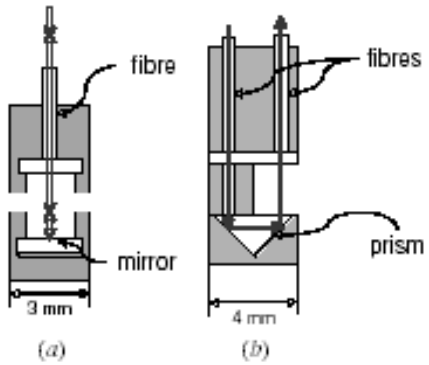


Fig. 90: (a) Immersion probe, with a single fibre and a fixed dimension analysis chamber. (b) Dual fibre plus prism immersion probe with an analysis chamber.

Noninvasive sensing

Noninvasive (noncontact) sensors are sensors which do not come in contact with the interior of the culture chamber, and are capable of measuring via interrogation through the bioreactor wall, for example by using ultrasounds or optical methods such as near-field optical microscopy (Fig. 91) spectrophotometry or fluorimetry, and chemical methods (Fig. 92).

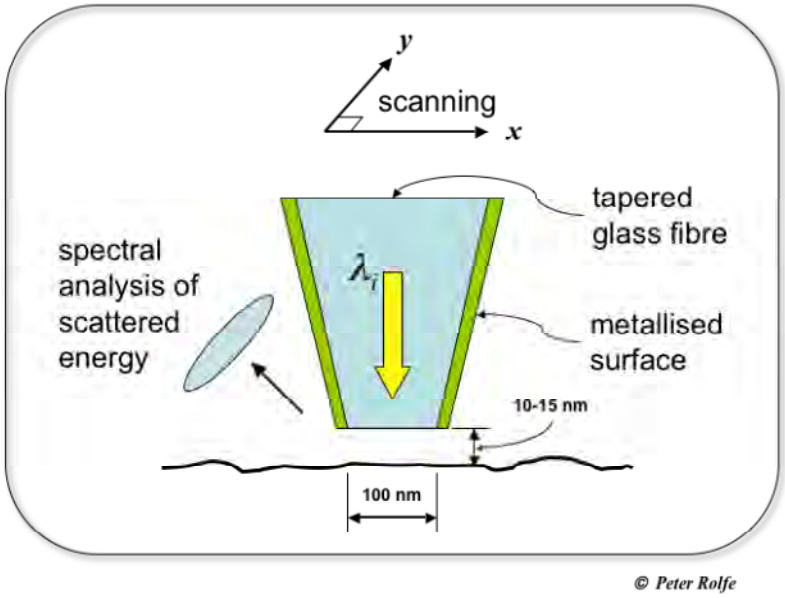


Fig. 91: A tapered glass fibre can be used in scanning near-field optical microscopy

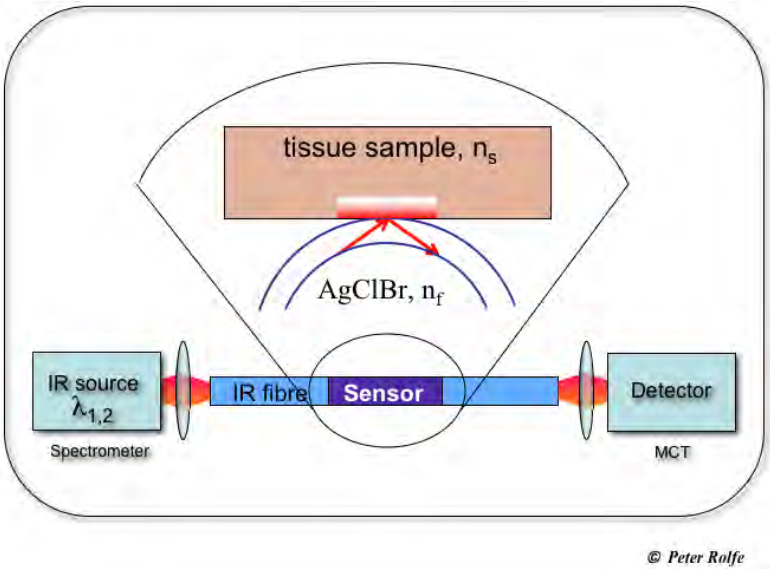


Fig. 92: Chemical sensing in tissues can be carried out using the principle of interactions with the evanescent wave.

This approach, while avoiding the sterility issues associated with invasive sensors, implies that the bioreactor wall must be either entirely or locally transparent to the investigating wave. Moreover, due to the presence of an intermediate material between the probe and the object of interest, it is more challenging to achieve high specificity and high sensitivity of the measurement with these sensors. Typically, the flow rate of medium in perfusion bioreactors is detected via noninvasive techniques, mainly based on Doppler velocimetry (Fig. 93). DOCT (Doppler Optical Coherence Tomography), for example, is a novel technique allowing noninvasive imaging of the fluid flow at micron-level scales, in highly light scattering media or biological tissues. Derived from clinical applications, DOCT has been adapted to characterize the flow of culture medium through a developing engineered vascular construct within a bioreactor chamber.

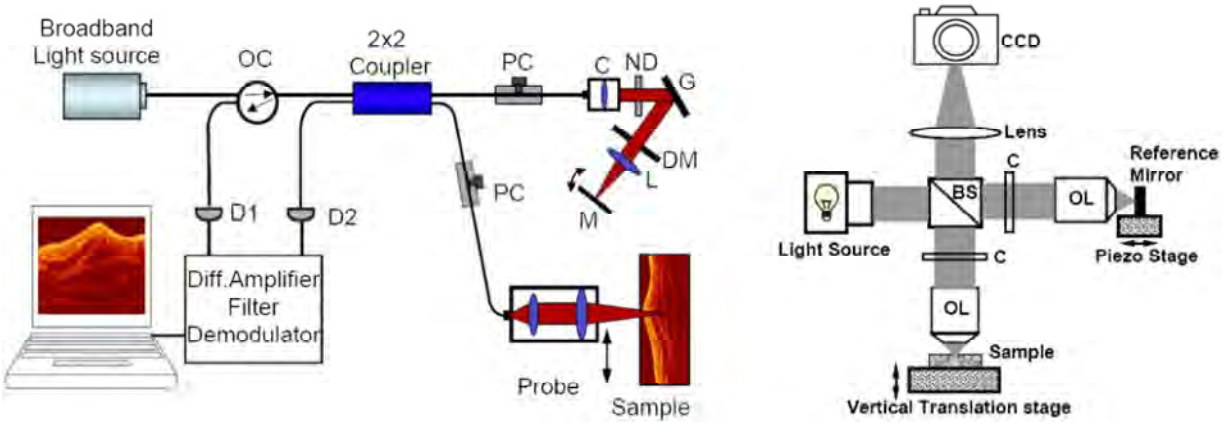


Fig. 93: Schematic of OCT system set-ups used for the current investigations. (a) Time-domain OCT where OC denotes the optical circulator, PC the polarization controller, C the collimator, ND the neutral density filter, G the grating, DM the double pass mirror, L the optical lens, M the reflecting mirror and D the detector. (b)Whole-field OCM where C denotes the compensators, OL the objective lenses and BS the beam splitter.

Indirect sensing

Indirect sensing is performed directly on the culture media, but via sampling means. The two main options included in this category are “offline analysis” and “shunt sensing.” In the first case, manual or automated online medium sampling is performed (with possible negative implications for the sterility of the closed system) and analyses are conducted with common instruments for bioanalytics (e.g., blood-gas analyzers).

In the latter, the measurement is directly carried out within the fluid, driven through a sensorized shunting loop and later either returned to the body of the bioreactor or discarded. Since probes do not need to be placed inside the culture chamber, indirect sensing can be performed by means of advanced, accurate instruments, with clear advantages in terms of specificity and sensitivity with respect to the invasive method. On the other hand, the lag-time introduced by sampling can heavily hinder the significance of the measurement itself (with possible introduction of artifacts) and impair the efficacy of feedback control strategies. With this method, pO_2, pCO_2, pH, and glucose concentration in the culture medium are typically measured; protein and peptide analysis can be also conducted via spectrophotometric and fluorimetric assays within shunting chambers.

Fig. 94, Fig. 95 and Fig. 96 show different indirect sensing methods.

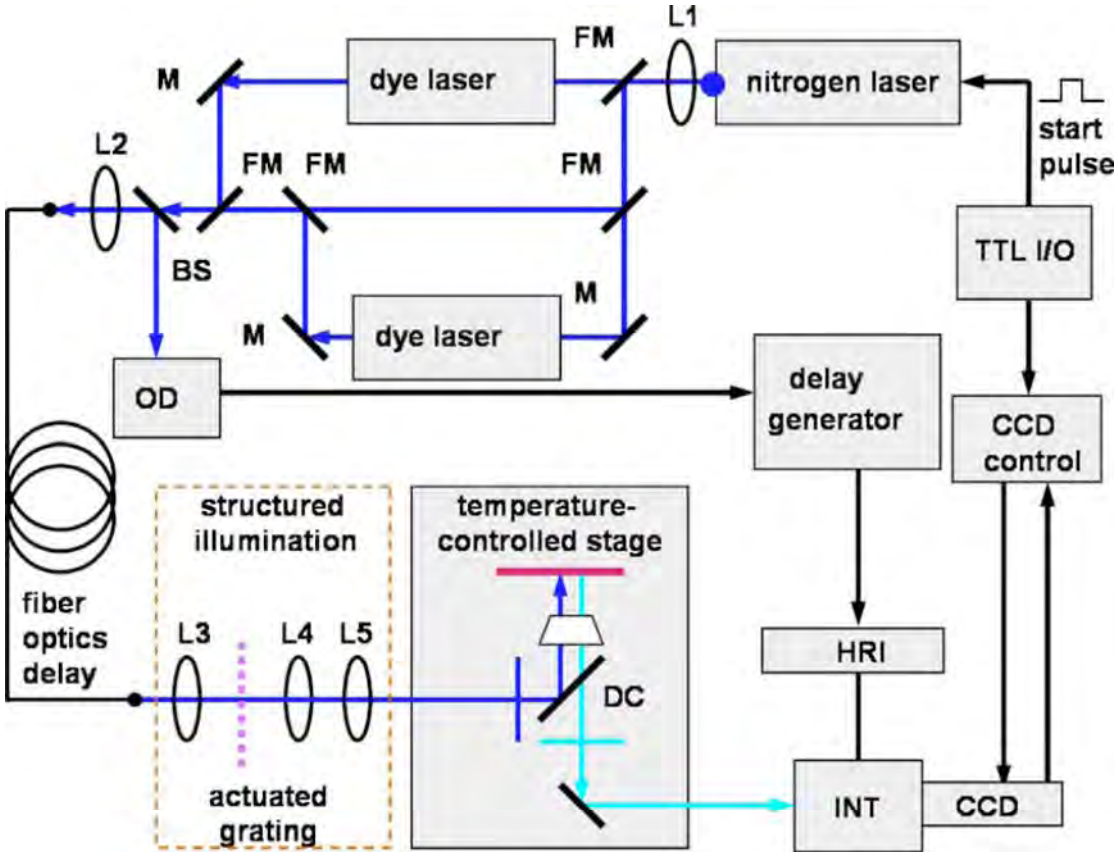


Fig. 94: Widefield, time-domain FLIM instrumentation: CCD, charge-coupled device; HRI, high-rate imager; INT, intensifier; TTL I/O, TTL _transistor-transistor logic_ input/output card; OD, optical discriminator; BS, beamsplitter; DC, dichroic mirror; FM, mirror on retractable "flip" mount; L1, L2, L3, L4, and L5, quartz lenses; M, mirror; colored thick lines are the optical path and black thick lines are the electronic path.

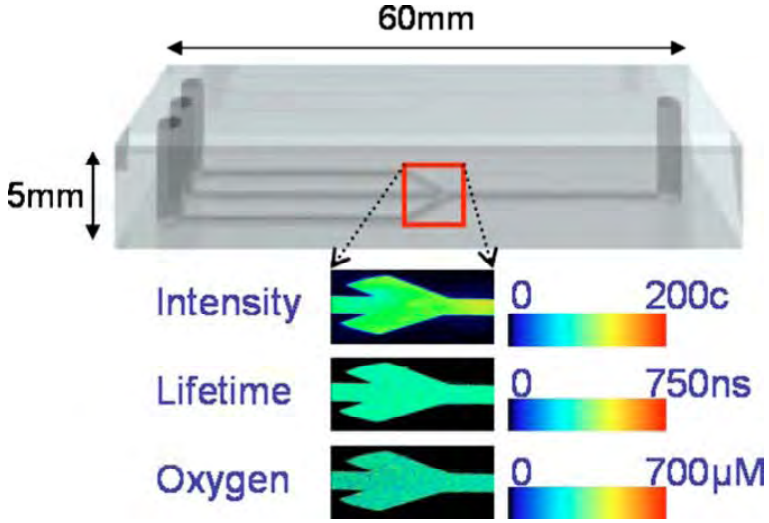


Fig. 95: Illustration of fluorescence intensity and lifetime imaging in microfluidic devices. Top: perspective view of a device that contained C2C12 mouse myoblasts and was perfused with media containing RTDP at a rate of approximately 0.5 nl/ s by gravitydriven flows. Channel height=50 µM, width=300 µm. Bottom: representative images of RTDP fluorescence intensity scale in counts, lifetime = microseconds, and oxygen M obtained via FLIM.

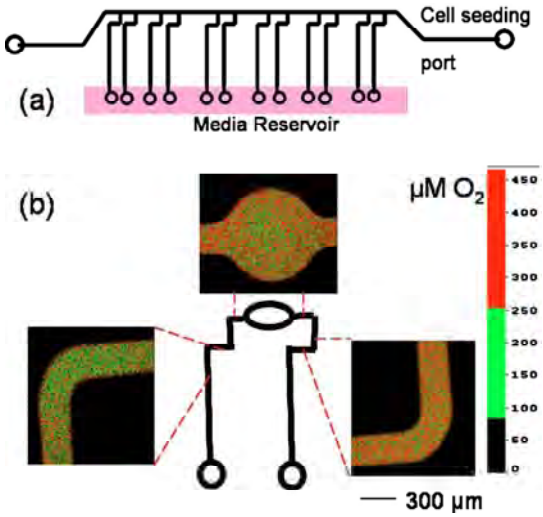


Fig. 96: Oxygen measurements from a closed-loop PDMS bioreactor for continuous cell culture of C2C12 mouse myoblasts. (a) Device schematic. Channel shape was an isosceles trapezoid with a height of 30 m and an upper (lower) PDMS layer of 180m (402 m). Each of the six loops has a right and left valve separating it from the others.(b) Oxygen distribution images at different points of a single loop (binary scale in M).

Monitoring of the Construct

While monitoring of the milieu is gradually entering the practice of bioreactorbased tissue engineering, monitoring the function and structure of developing engineered constructs still remains a relatively uncharted area and a highly challenging field of research. In this application, it would be limiting to use the term “sensor” in the traditional sense, since the techniques currently under study are based on highly sophisticated cutting edge technology, often inherited from rather unrelated fields (e.g., clinics, telecommunications). Systems for the nondestructive online monitoring of the construct developmental state would allow continuous and immediate optimization of the culture protocol to the actual needs of the construct itself, thus overcoming the drawbacks traditionally related to the use of endpoint detection methods or fixed time point analyses. Typically, research is being driven by the need for real-time characterization of (i) functional and (ii) morphological properties of engineered constructs, both at the

micro- and at the macro-scale. The following paragraphs give a brief overview of the most recent techniques applied in bioreactors for these cited purposes.

Monitoring of the functional properties

Monitoring of the functional properties comprises characterization of the construct 's overall physical properties (e.g., strength, elastic modulus, permeability), but also monitoring of cell function within the engineered construct itself, e.g., in terms of proliferation, viability, metabolism, phenotype, biosynthetic activity, and adhesive forces. In this context, Stephens and collaborators (234) recently proposed a method to image real-time cell/material interactions in a perfusion bioreactor based on the use of an upright microscope (Fig. 97).

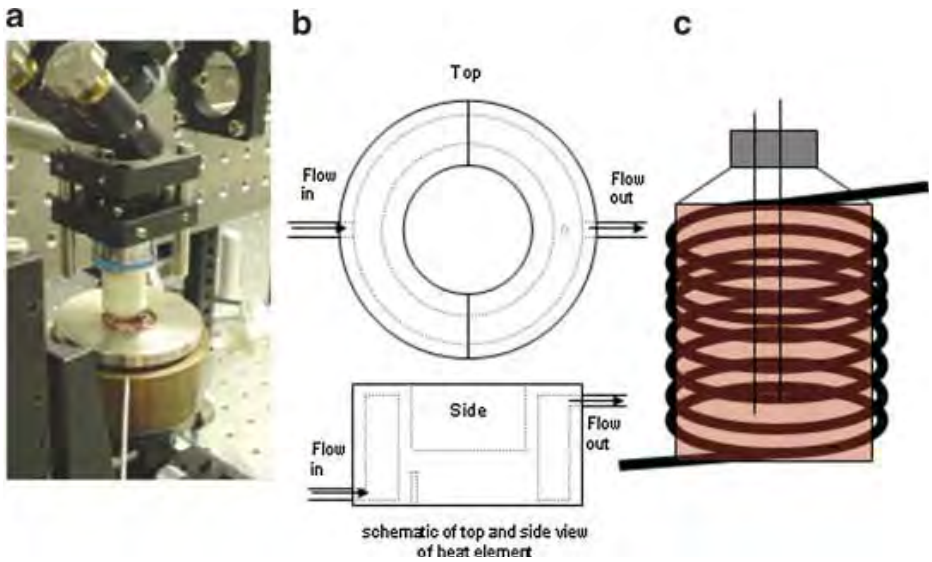


Fig. 97: Heating system, (a) image of bioreactor in heating element on the microscope stage, (b) top and side view of copper water jacket machined to hold the bioreactor; and (c) pliable copper tubing was wrapped tightly around a 250 mL reservoir bottle and covered with insulation. Both heating elements were attached to the circulation bath (378C) and the water was used to warm the medium and bioreactor (234).

The kinetics of cell aggregation and organoid assembly in rotating-wall vessel bioreactors, instead, could be performed according to the method developed by Botta et al. (235), relying on a diode pumped solid state laser and on a CCD video camera. Boubriak and co-authors (236) recently proposed the use of micro-dialysis for detecting local changes in cellular metabolism (i.e., glucose and lactate concentrations) within a tissue-engineered construct. By means of this method, concentration gradients could be monitored within the construct, with the highest lactate concentrations in the construct center, thus allowing early detection of inappropriate local metabolic changes.

Monitoring of the morphological properties

Monitoring of the morphological properties of engineered constructs essentially encompasses assessing the amount, composition, and distribution of the extracellular matrix which is being deposited throughout the scaffold during bioreactor culture. Monitoring morphological properties is particularly pertinent when engineering tissues whose function is strictly dependent on the structural organization of their extracellular matrix, e.g., bone and tendons. In this context, OCT (Optical Coherence Tomography, Fig. 98) has been successfully employed as a real-time, nondestructive, noninvasive tool to monitor the production of extracellular matrix within engineered tendinous constructs in a perfusion bioreactor.

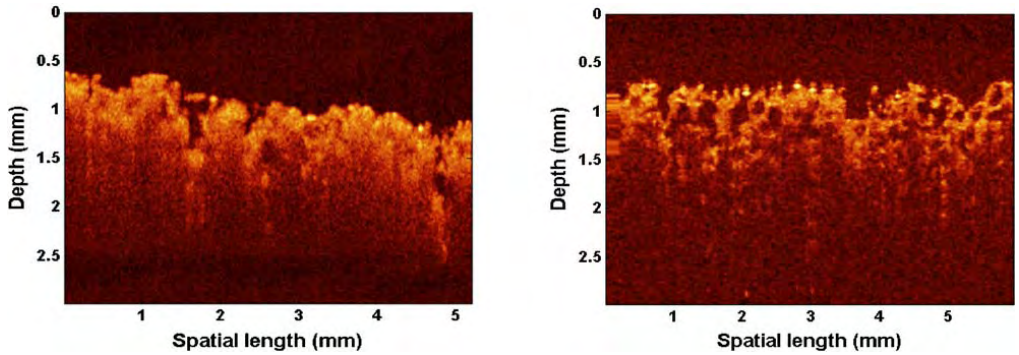


Fig. 98: TD-OCT images of the scaffolds seeded with 1 × 10^6 bone cells and cultured for 1 week: (a) scaffold incorporated with TCP particles and (b) cells adhering beads.

OCT is analogous to conventional clinical ultrasound scanning (scheme in Fig. 99), but using near infrared light sources instead of sound, it enables higher resolution images (1–15 µm vs. 100–200 µm); the technique is compact and flexible in nature, as well as relatively low cost since it can be implemented by commercially available optic fibres.

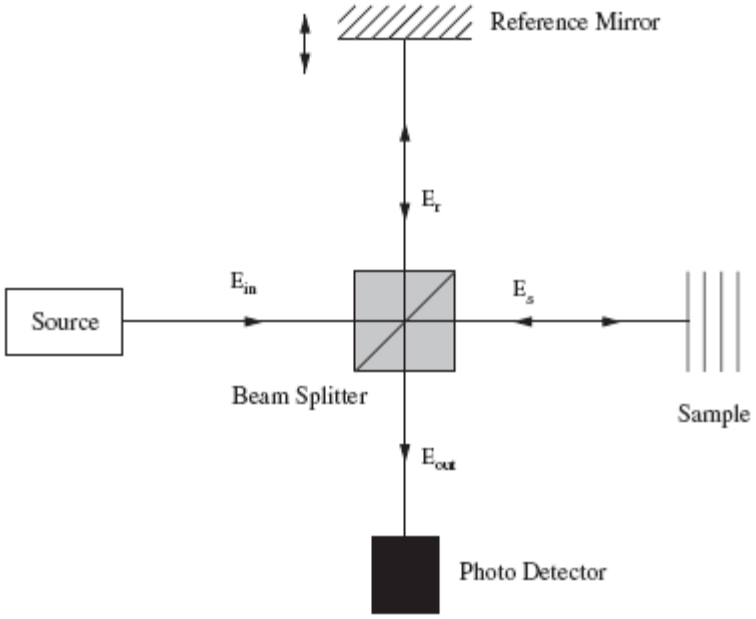


Fig. 99: Basic OTC system.

However, the most promising technique in the field of real-time imaging is undoubtedly µ-CT (micro-computed tomography). Using this technique, for instance, the mineralization within a three-dimensional construct cultured in a perfusion bioreactor was monitored over time, allowing quantification of the number, size, and distribution of mineralized particles within the construct (Fig. 100 and Fig. 101).

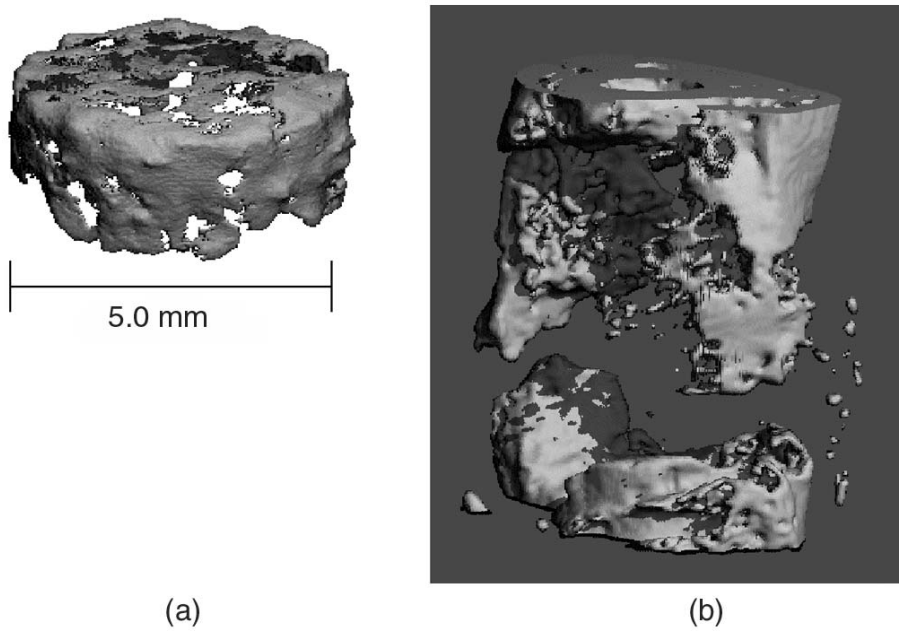


Fig. 100: Mineralized matrix formation within a 3-D construct composed of a polymeric scaffold and marrow-derived progenitor cells following 28 days of culture in osteogenic media (a). Bone formation within a polymeric scaffold implanted into a rat critical-size (5 mm) femoral defect (b).

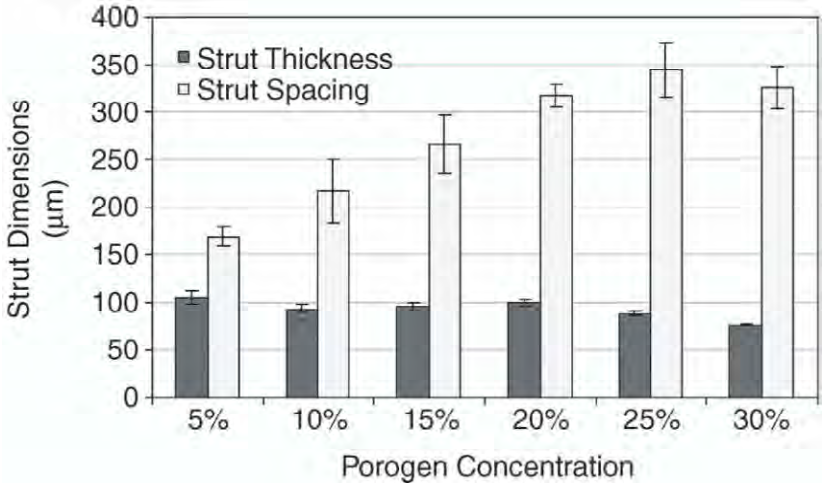


Fig. 101: Micro-CT-based microarchitectural parameters as a function of porogen concentration for porous poly(L-lactide-co-DL-lactide) (PLDL) scaffolds.

We have underlined how progress made in the in vitro generation of 3D tissues starting from isolated cells is slowed down by the complexity of the process and of the interplay among different culture parameters. The establishment of well-defined and controlled bioreactor-based 3D culture model systems, supported by modeling efforts and sensing technologies, will be key to gain a deep insight into the mechanisms of tissue development at the research level and, consequently, may provide an advanced technological platform for the achievement of more applicative, highthroughput objectives, e.g. enabling drug screening and toxicology studies, fostering the development of new, rational design criteria for advanced biomaterials/implants, as well as allowing functional quality control of engineered tissues. Besides limiting the recourse to complex, costly and ethically questionable in vivo experiments in animal models, such an approach would found the basis for safe and standardized manufacture of grafts.

The culture of cells in the various configurations of bioreactor to produce tissue constructs presents many challenges for those developing sensors and instruments for monitoring the key chemical and physical variables. The bioreactors range from the simplest, comprising glass vessels containing cells in suspension, through to those in which cells attached to or enclosed by 3D scaffolds are subjected to controlled mechanical stimuli such as cyclic axial load or shear stress. For fundamental research studies complex cell and tissue culture apparatus, including specialized microscopes, spectrophotometers, fluorimeters, DNA microarrays and biosensor chips, can be justified. On the other hand, in the longer term the large-scale fabrication of tissues suitable for routine clinical use will require simplified monitoring apparatus that will nevertheless provide a sufficiently precise and accurate temporal indication of the key bioreactor processes. Invasive sensing, for example with the electrochemical or fibre optic sensors described here, can undoubtedly provide the most direct measurements of chemical and physical variables relevant to bioreactor processes. The insertion into the bioreactor requires that the sensors are amenable to sterilization and this is not necessarily easy to achieve, given the specialized optical components or diffusion membranes. The sensors described here, and others, require initial calibration and this needs to be repeated periodically. Calibration drift occurs sometimes due to protein adsorption on the sensor membrane surfaces which changes the analyte diffusion processes and thereby the sensor sensitivity and response time. The design of the sensors should allow easy insertion and removal from the bioreactor without compromising sterility. Sensors' lifetime may need to be of several weeks, unless they are sufficiently low cost that they may be disposed of and replaced. In some situations the use of the sampling shunt provides good opportunities for comprehensive analysis of bioreactor contents, using printed sensor arrays or optical analysis or indeed combinations of both. The sampled contents, however, need to be defined carefully before a particular shunt design is used. When one is dealing with culture fluid without suspended cells then micro-fluidic chambers can be used without too much risk of channel blockage by cells or cell fragments. In the case of cell suspensions, or even cells adhered to carrier beads, larger sample channels are obviously needed. One method to overcome some of these problems is to have means for particulate/cell filtering or separation, so that the chemical analysis is essentially carried out with a simple fluid sample. The use of separation or dialysis membranes is being considered here as part of the measurement strategy. NIR spectroscopy appears to offer several real possibilities for cell and tissue bioreactor measurement. The earlier work on so-called at-line NIRS for monitoring microbial bioprocesses and in situ NIRS for monitoring key analytes in mammalian cell culture has indicated good prospects for measurement of glucose, lactate, glutamine and ammonia.

It is possible that immersion optical probes may be suitable for direct tissue bioreactor monitoring and the shunt most certainly can be used for this approach. Protein structure and function can undoubtedly be studied with spectroscopy and this may be valuable in answering fundamental questions related to specific aspects of cell or tissue culture. Absorbance and fluorescence measurements of Trp in the UV bands require careful control due to the environmental influences on absorbance peaks and emission intensity. Trp fluorescence lifetime determinations (typically 5–10 ns) could be valuable in the study of quenchant influences and of energy transfer processes. The most important chemical species to be monitored for bioreactor control are dissolved oxygen, glucose, pH and PCO2. The invasive devices used here appear to have adequate performance for this application. Glucose biosensors may also be fabricated by electrode position using layer-by-layer techniques.

In addition to these basic chemical variables it may well be that detection of certain proteins synthesized by the cells being cultured could help in the process of optimizing tissue fabrication. An example of this is in

the research on vascular endothelial cells (VECs) which involves the study of the important vasodilator prostacyclin (PGI2). Prostacyclin measurement with bioluminescence has been reported.

Hypoxia is thought to trigger the production of prostacyclin and the measurement of PO2 and PGI2 could help to unravel the precise mechanisms.

Optical immuno-biosensors using the surface plasmon resonance could also provide opportunities for analysis of more complex markers of cell and tissue behaviour. In the longer term there may be prospects for applying process control strategies to tissue bioreactors. Fermentation for industrial as well as pharmaceutical products has been the subject of control interest. For example, genetic algorithms have been applied to parameter estimation in Escherichia coli fermentation.

2.3. Basic bioreactor design and development

The bioreactor design activity was first carried out inside BIOSCENT ("BIOactive highly porous and injectable Scaffolds controlling stem cell recruitment, proliferation and differentiation and enabling angiogenesis for Cardiovascular ENgineered Tissues"), a FP7 European project, whose overall objective is the development of innovative bioactive polymeric scaffolds able to guide tissue formation from dissociated stem cells, for engineering autologous cardiovascular replacements, namely vascular tissues, heart valves and cardiac muscle, and for cardiovascular repair, namely cardiac injectable gels.

Within this project, Work Package 4, that involved Politecnico di Torino, Sorin Biomedica Cardio, University of Pisa, University of Parma and Petru Poni Institute of Bucarest, had as main objectives to design, realise, and deliver bioreactors for in vitro guiding tissue formation for engineering autologous cardiovascular replacements and to provide variants in bioreactors depending upon the type of tissue to be cultured.

Particularly, the research activity, limited to cardiac muscle and cardiac injectable gel bioreactors, involved:

- assessment of user requirements and definition of technical specifications;
- definition of the architectural design of the dynamic culture devices (mechanics, chemical/fluids, controls, sensors, actuators, operator/computer interface);
- system detailed design;
- definition of system operation (sterilisation, sensor/actuator calibration, culture feeding, culture stimulation, visualisation and data acquisition);
- realisation of bioreactor prototype components/subsystems;
- realisation and assembly of bioreactor prototypes;
- behaviour/operation testing of bioreactor prototype components/subsystems (bioreactor in house factory tests).

As long as any construct requires defined stimuli with respect to the specific cell lineage and to the scaffold physical response, two different bioreactors have been designed to engineer two different constructs, namely cardiac muscle and cardiac injectable gel. This choice has been adopted to deliver custom parameters to enhance cell proliferation/differentiation of the specific cell lineage through specific chemical/physical stimulation to be applied within the bioreactor and to monitor only specific parameters with relevant significance for the in vitro cell culture system.

In order to identify these specific parameters, a preliminary user requirement analysis has been done with final definition of the product specifications.

2.3.1. User requirement definition: User Requirement Document (URD)

An user requirements document (URD) is a document usually used in engineering that specifies the requirements the user expects from the device to be constructed in a project.

An important and difficult step of designing a product is determining what the end user actually wants it to do. This is because often end users, not being technicians, are not able to communicate the entirety of their needs and wants, and the information they provide may also be incomplete, inaccurate and self-conflicting. The responsibility of completely understanding what the end user wants then falls on the designer of the product. Once the required information is completely gathered it is documented in a URD, which is meant to spell out exactly what the product must do and becomes part of the contractual agreement.

Within the BIOSCENT - WP4 activity, a User Requirement Document was needed to start the design activity of BIOSCENT bioreactors; this document offered to BIOSCENT partners a list of requirements/issues related to the devices to be realised.

This document, that was elaborated in cooperation with Politecnico di Torino and Sorin Biomedica Cardio, is attached (ANNEX I).

2.3.2. Realisation of Dynamic Culture Device Prototypes

Two different bioreactors where designed and realised.

The bioreactor for cardiac muscle was named “Cardiac Patch Dynamic Culture Device (CPDCD)”. Modifying an old prototype conceived to stimulate skeletal muscle bundles (237), it was possible to obtain a device suitable for BIOSCENT purposes.

The bioreactor for cardiac injectable gel was named “Hydrogel Dynamic Culture Device (HDCD)”.

Cardiac Patch Dynamic Culture Device (CPDCD) Prototype

The cardiac patch dynamic culture device (CPDCD) prototype is designed to test cardiac constructs based on cells seeded on the BIOSCENT cardiac patches.

Technical requirements for CPDCD

Using the information gathered by the URD (ANNEX I), technical requirements of the device were assessed as follows.

Cell type

Cell lines the CPDCD grows:

- Human Umbilical endothelial cells (HUVECs) (as a lining for antithrombogenic properties)
- Human bone marrow mesenchymal stem cells (MSCs) (as a SMCs source if appropriated differentiation protocols are defined)
- Human adipose derivated stem cells (ASCs)
- Mouse/rat Bone marrow MECs (as a SMCs source if appropriated differentiation protocols are defined)
- Mouse embryonic stem cells (for testing, proof of concept)

Stimulation and perfusion

BIOSCENT cardiac patch requires mechanical and electrical stimuli.

A static culture medium is needed during seeding phase, then perfusion is required while culturing.

Performance requirements

Stimulation and perfusion	
Parameter	**Value (or range)**
Stress [Pa]	0.06 – 0.15
Strain [%]	10 - 20
Stimulation voltage [V]	0.01 - 3
Stimulation electrical field [V/mm]	0 – 0.8
Perfusion flow [mm^3/s]	5.3 - 25
Perfusion pressure [mmHg]	80 – 200 (±30)

Measurement	
Parameter	**Value (or range)**
Temperature [°C]	37
Dissolved CO_2 [mmHg]	48 - 55
Dissolved O_2 [mmHg]	115 - 130
Glucose consumption rate [g/l/day]	2.216±0.02
Lactate consumption rate [g/l/day]	1.436±0.12
Glutamine rate [mM]	2 – 4
pH	7.21 – 7.33

Design requirements	
Feature	**Requirement**
Number of constructs supportable	Any
Culture chamber	Acrylic, maximum volume 300 ml
Spatial orientation of constructs	Horizontal

Priming volume [ml]	30

Architectural design

The general functions of the CPDCD prototype are: (i) the control and the delivery of accurate and reproducible combined physical stimuli, i.e., stretching and electrical patterns according to the technical specification edited by BIOSCENT partners; (ii) the generation of a biochemical environment suitable for growth and differentiation of cells cultured on cardiac patch scaffolds developed in BIOSCENT project.

Table 4: Mechanical/electrical stimulation specifications.

Stimulation	
Parameter	**Value (or range)**
[Pa]	0.06 – 0.15
Strain [%]	10 – 20
Stimulation voltage [V]	0.01 – 3

Key constitutive elements of the CPDCD prototype are (Fig. 102):

- a transparent, sealable and sterile culture chamber where cells, seeded on polymeric scaffolds, will be housed and submerged in culture medium during the entire duration of the experiments;
- a mechanical stimulation subsystem, including grips, stepper motor and controller, that provides predefined uniaxial tensile cyclic loading to the biological constructs;
- an electrical stimulation subsystem, including electrodes and voltage source, that provides predefined electrical stimulation to the biological constructs (yet designed, implementation in progress);
- a recirculation subsystem, constituted by oxygen-permeable tubes, a peristaltic pump, a fresh media reservoir, and a waste receptacle.

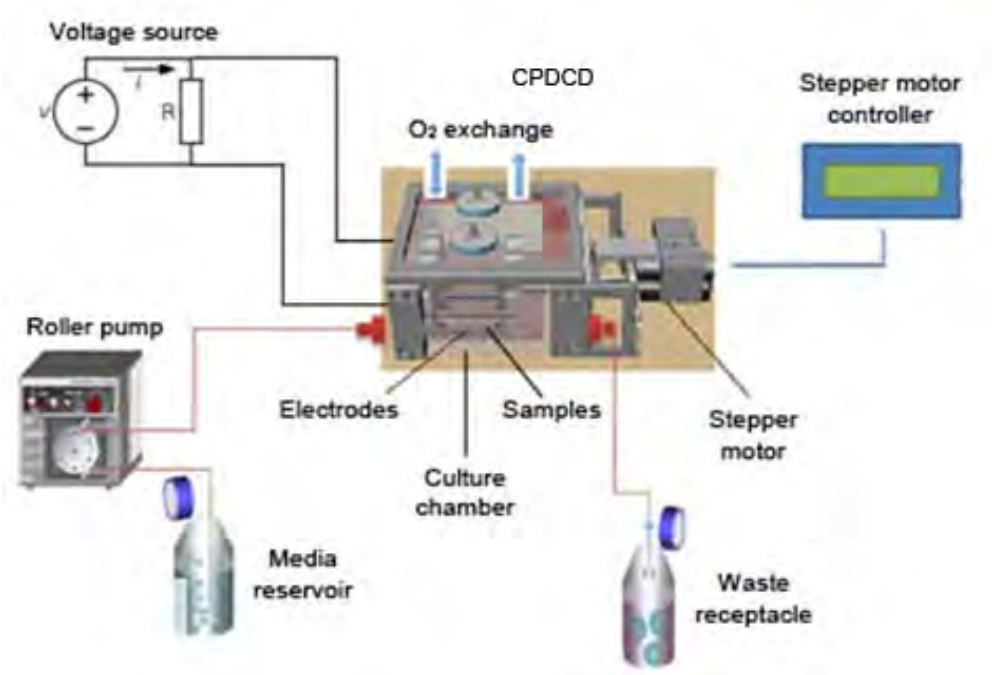


Fig. 102. Architectural design of CPDCD constituted of: a culture chamber; a mechanical stimulation subsystem; an electrical stimulation subsystem; a recirculation subsystem.

The CPDCD prototype is characterized by a high versatility of the mechanical/electrical stimulation, allowing the device to be employed with several constructs and for different applications. In particular, its technical features make it suitable for the cultivation of cells for cardiac tissue engineering. It is also valuable to assure full compatibility with GLP procedures. The sterility maintenance of the culture was specifically taken into consideration during the device conception.

Moving from these general design criteria, the CPDCD prototype was realized by satisfying the following constraints and requirements:

- cytocompatibility of all the materials in contact with culture medium;
- corrosion-resistance of the materials in contact with culture medium;
- ease of sterilization and sterility maintenance;
- ease of use (assembly in sterile conditions under a laminar flow hood, medium exchange, cleaning, use for non-trained staff);
- small dimensions, suitable for positioning in a cell culture incubator;
- no medium stagnation during exchange operations;
- housing of an experimentally significant number of specimens.

Detailed list of components

Culture chamber

The culture chamber (Fig. 103) of the CPDCD prototype, i.e., the sterile and cytocompatible environment where cell are cultured in, has been manufactured through material removal by a micrometrical controlled cutter from a Poly(methyl methacrylate) (PMMA) bulk piece, a choice that guarantees from the obtainment of transparency. The inner dimensions are approximately 100x97x55 mm. The rounded edges were

conceived in order to avoid stagnation points and no discontinuities, fissures, interstices, holes, which are preferable targets for microbial contamination.

In the chamber, two groups of four grips, each of them screwed onto a crossbar, are positioned symmetrically to hold four constructs. To mechanically stimulate the samples, one crossbar is fastened to the culture chamber inner wall, while the other is screwed to a couple of drive shafts connected to the stepping motor. A couple of dynamic diaphragms enwraps each drive shaft assuring sterility and allowing motion transmission. An AISI 316 rank provides a fastening structure for the stepping motor and for four toggle latches, constituting at the same time two symmetrical handling points. The chamber is equipped with a lid, on the top of which two oxygen filters are located, that is kept in position by toggle latches.

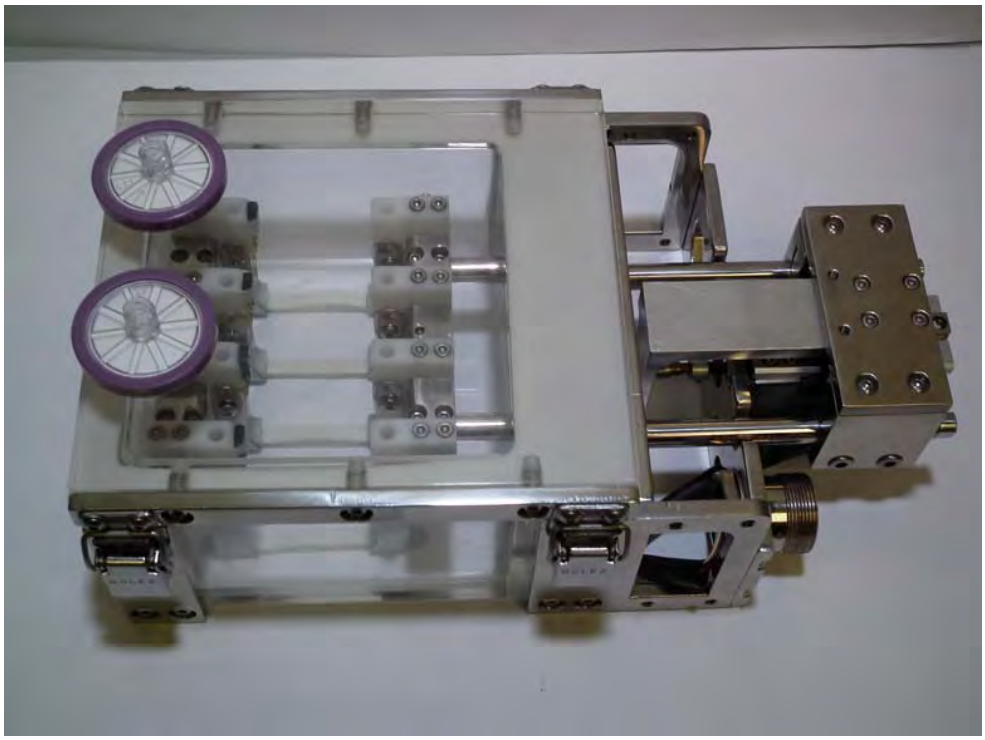


Fig. 103. CPDCD prototype.

As for the electrical stimulation, a dedicated subsystem has been designed (Fig. 104): five AISI 316L electrodes can be placed parallel to the constructs and in alternate position (with alternate polarity) in order to provide electrical field distributions to the cell-seeded samples. An external electrical stimulator provides different voltage-controlled stimulation output signals.

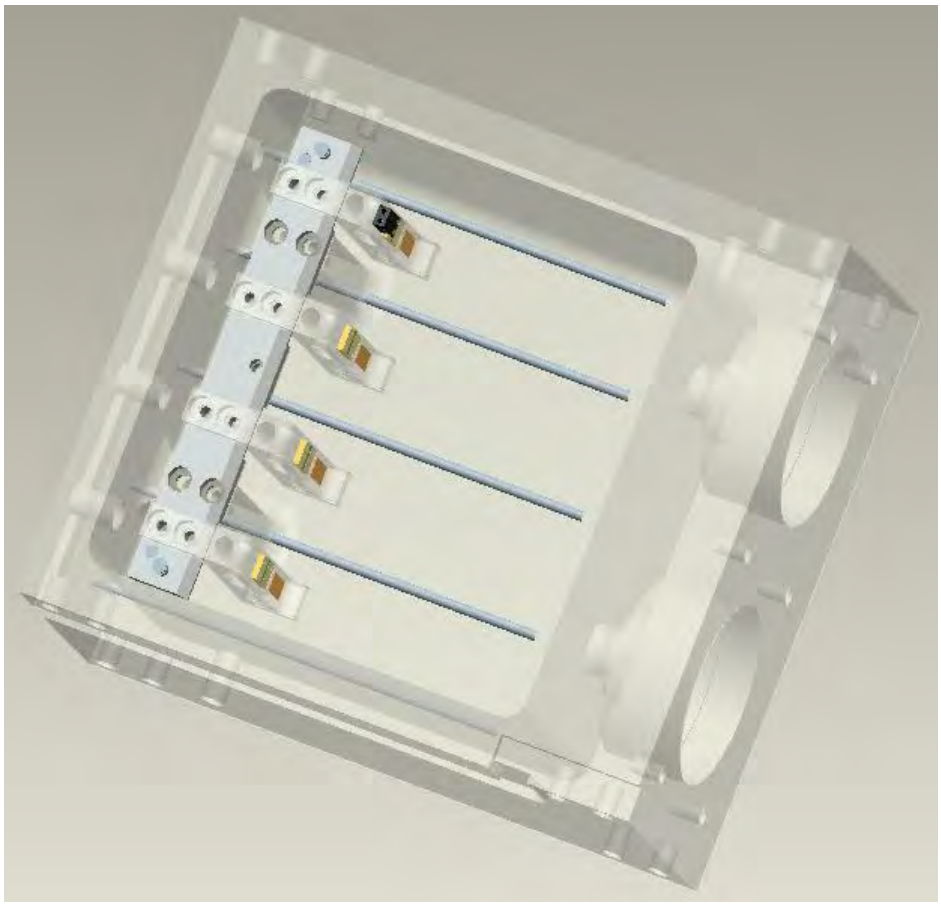


Fig. 104. Design of the electrodes' position within the CPDCD prototype.

Mechanical stimulation subsystem

The mechanical stimulation subsystem (Fig. 105) is made of a 5 phase stepping motor driving two drive shafts screwed onto a crossbar that fixes four mobile grips for the samples. The dynamic diaphragms that enwrap each drive shaft assuring sterility permit an 8 mm maximum uniaxial dislocation.

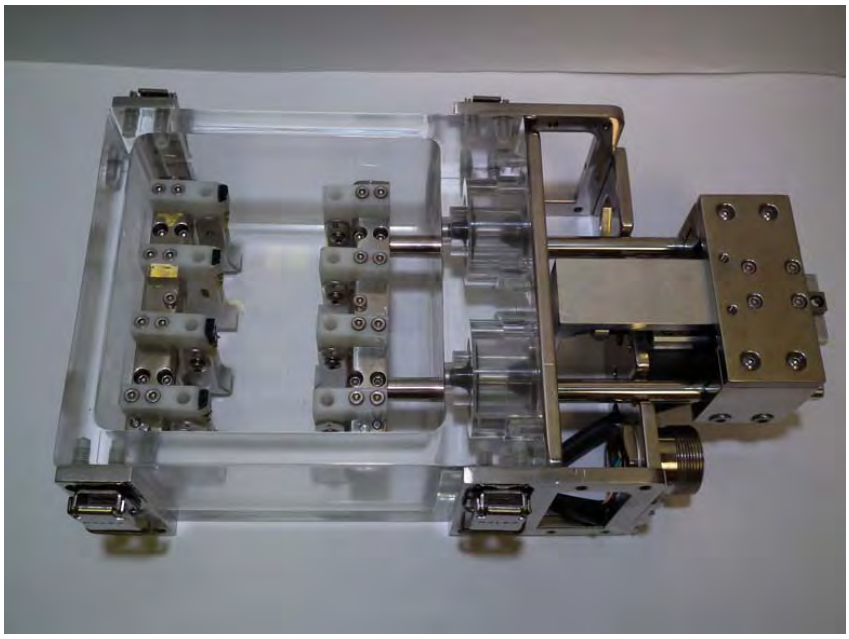


Fig. 105. CPDCD prototype mechanical stimulation subsystem.

Motor

The micrometrical stepper motor (DRL28PB1G-03, LIMO series, Oriental Motor, Torrance, Ca, USA, Fig. 106), develops a maximum holding force of 30 N, has a 2 µm resolution single step with a maximum acceleration of 0.2 m/s^2 and a maximum speed of 24 mm/s. Nominal stimulation frequency limit is 6 Hz (stepper motor features are reported in Table 5). Further details about the reasons of this choice and principles of stepper motors can be found in the BIOSCENT Deliverable 4.1.

Fig. 106. DRL28PB1G-03 stepper motor (by Oriental Motor©)

Table 5. DRL28PB1G-03 stepper motor specifications.

Parameter	Value (or range)
Number of phases	5
Driving mode	Half-step
Resolution single step	0.72°-4 µm
Resolution half step	0.36°-2 µm
Maximum stroke	8 mm
Maximum speed	24 m/s
Maximum acceleration	0.2 m/s^2
Encoder	Not present

The stepper motor is fixed to the culture chamber by means of a screw frame and connected with shafts by a holding structure(Fig. 107).

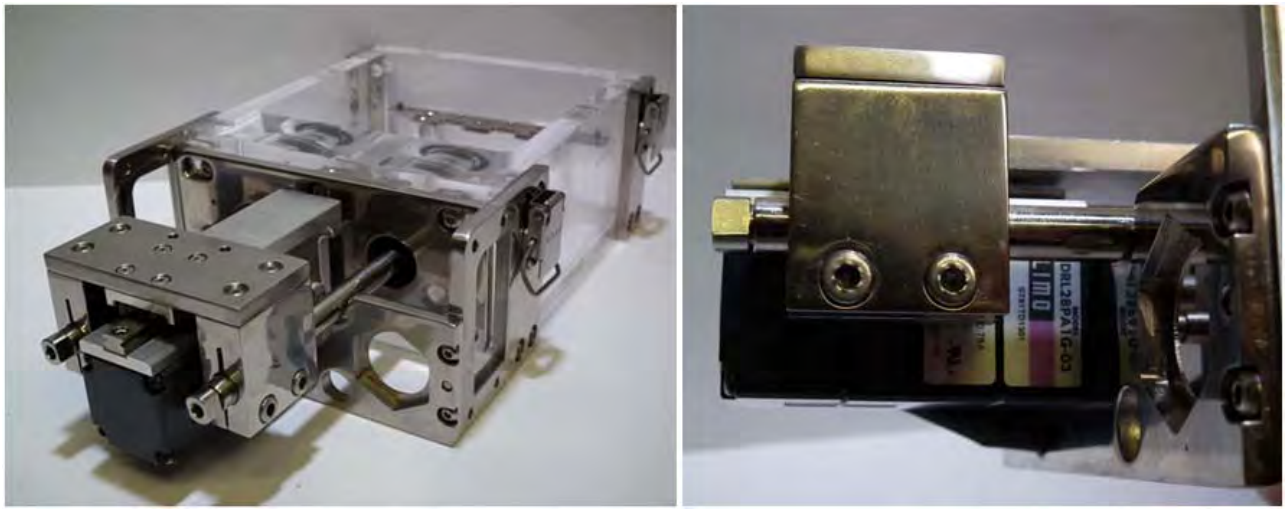


Fig. 107. Stepper motor connection to CPDCD prototype (left) and stepper motor detail (right).

Grasping system

Four couples of grips (Fig. 108) were designed to satisfy both the holding and the preservation of the integrity of the specimen during the experiment. The grip bodies have been obtained from Polyoxymethylene (POM) and a silicone sheet is placed on their bottom as an absorber, while the mobile component has been manufactured from an AISI 316L plate.

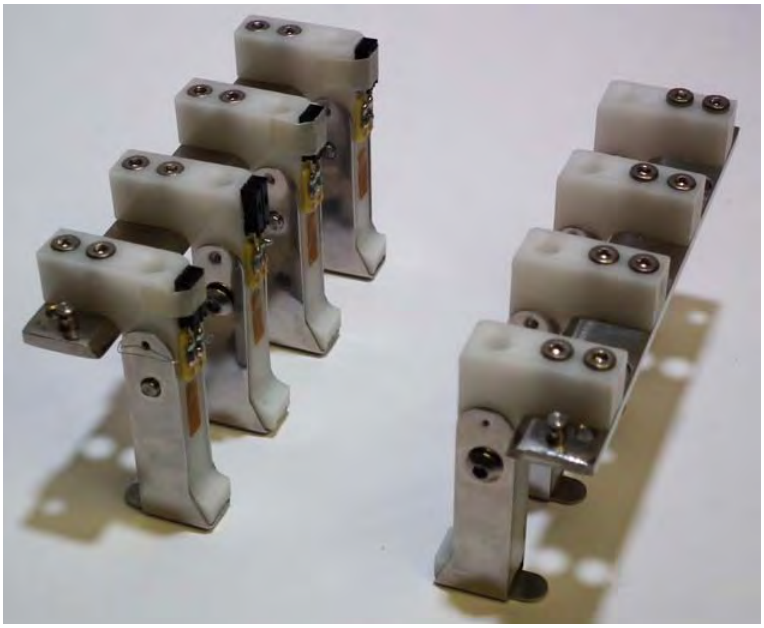


Fig. 108. CPDCD prototype grips.

The grasping system (Fig. 109) is based on a compression system that pulls the mobile component (clamp) against the grip body assuring a controlled holding pressure (around 100 Pa). This pressure is transferred uniformly to the biological constructs, avoiding load concentration points due to two silicon sheets, positioned on the mobile component.

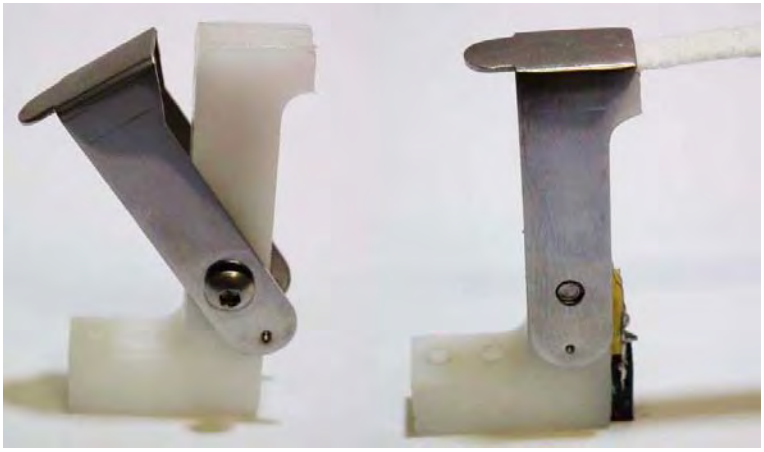


Fig. 109. CPDCD prototype grip system in open (left) and closed (right) position.

For the sake of convenience and ease of use, the first version of CPDCD prototype grips, presented in BIOSCENT Deliverable 4.1, was re-designed (Fig. 110). The second version of grips, made of PMMA, was

built and used in preliminary tests, but a tendency to fracture was noticed in a specific region of the base of the grip (Fig. 111).

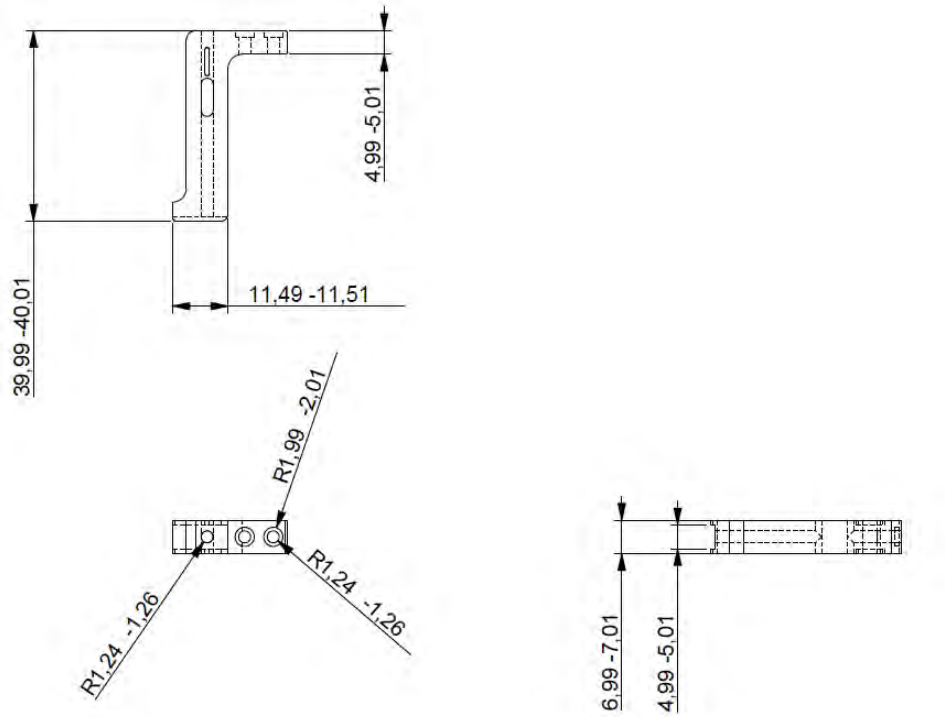


Fig. 110. Design of second version of CPDCD prototype grips (overall non refined dimensional drawing).

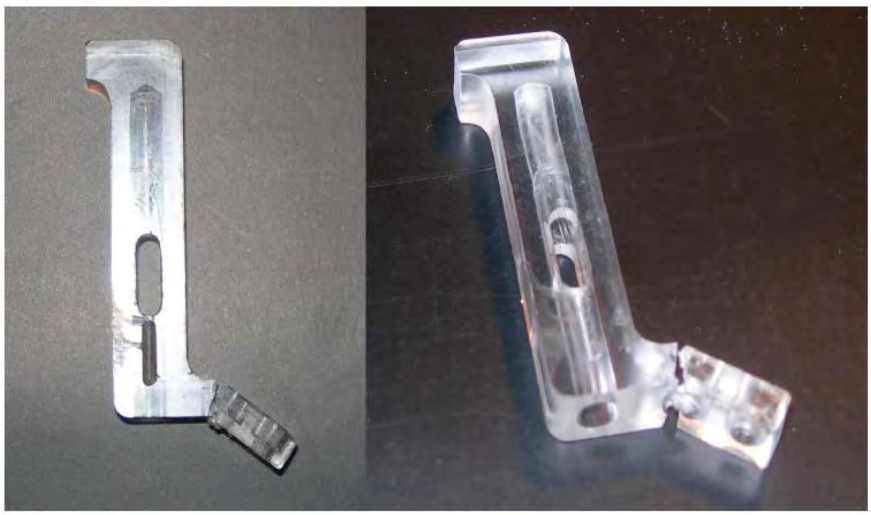


Fig. 111. Broken CPDCD prototype grip.

To get insight into the reason of this unexpected behaviour, using the properties of PMMA reported in Table 6 a FEM model has been created to simulate the behavior of the grip under load. FEM simulation features are reported in Table 7.

Table 6. PMMA properties.

Feature	Test Method	Units	PMMA XT
Density	DIN 53479	g/cm3	1,19
Tensile strength	DIN 53455	N/mm2	72
Elongation at break	DIN 53455	%	4,5
Young's modulus	DIN 53455	N/mm2	3200
Bending strength	DIN 53452	N/mm2	105
Bending modulus	DIN 53452	N/mm2	2900
Brinell hardness H961/30	DIN 53456	N/mm2	190
Compression strength	DIN 53454	N/mm2	103
Charpy unnotched impact strength	ISO179 -DIN 53453	kJ/m2	12
Charpy notched impact strength	ISO179 -DIN 53453	kJ/m2	2
Water absorption 24h	DIN 53495	%	0,3

Table 7. FEM simulation parameters for CPDCD prototype grip analysis.

Number of elements of the mesh	708 tetras, 1218 edges, 1664 faces
Method	Multi-pass adaptive
Polynomial order	2 to 7
Percent convergence	25
Converge on	Local displacement and local strain

Constraints have been imposed on the contact point between grip and crossbar, while force is oriented in y-direction and applied on the upper part of the grip (Fig. 112).

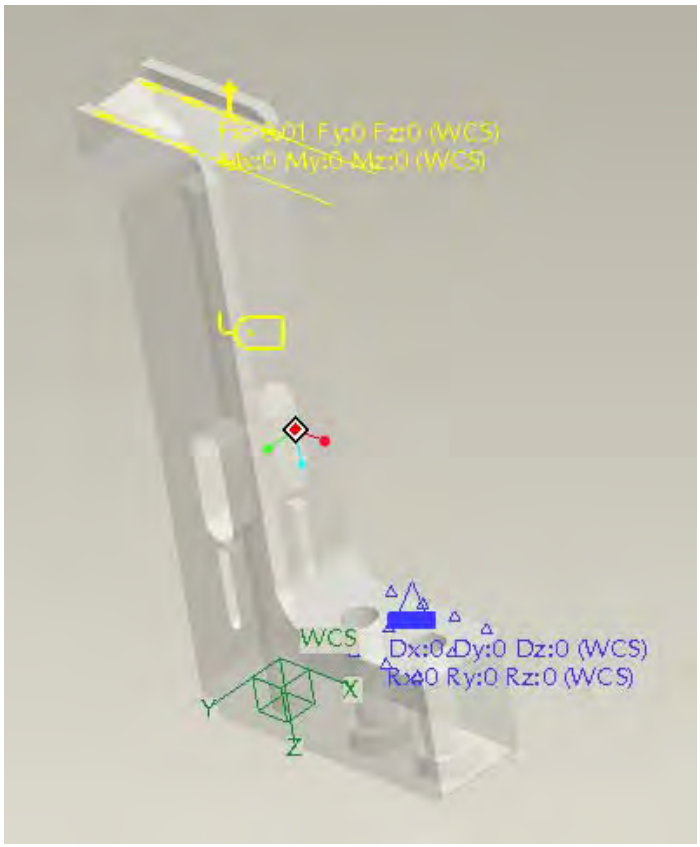


Fig. 112. Constraints and load condition in FEM simulation of CPDCD prototype grip.

The FEM analysis highlighted the presence of a concentration of in the area (that will be named “base of the grip” or simply “base”) were the fracture took place (Fig. 113). The fracture can probably be connected with a fatigue phenomenon.

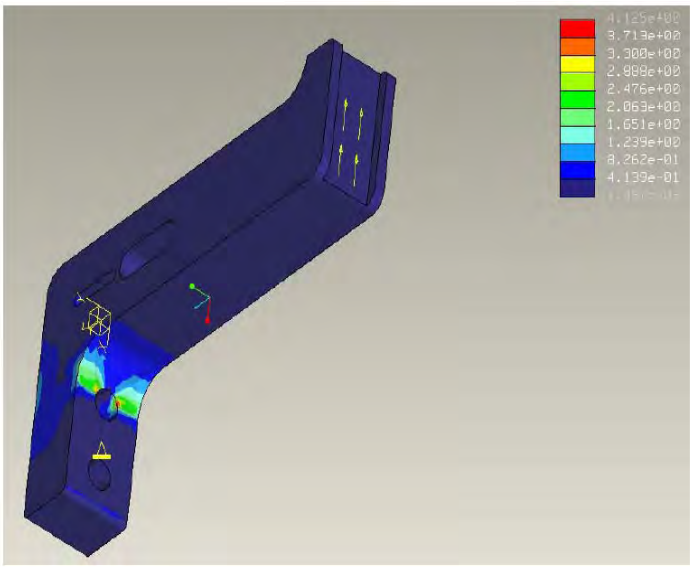


Fig. 113. Von Mises in CPDCD prototype grip under 1 N load.

The idea of redesigning grips was kept under consideration with the aim of adapting the grip geometry to fulfil the potentiality of the system. The stepper motor used in CPDCD prototype (DRL28PA1G-03 by Oriental Motor) is capable to exert 30 N force as its maximum value. So, the grip should bear a 30 N shear load at its worst condition. It was proved by a FEM simulation that the grip breaks under maximum load because Von Mises at the base covers a range of 60-180 MPa, a quantity that overcomes PMMA's tensile strength limit (Fig. 114).

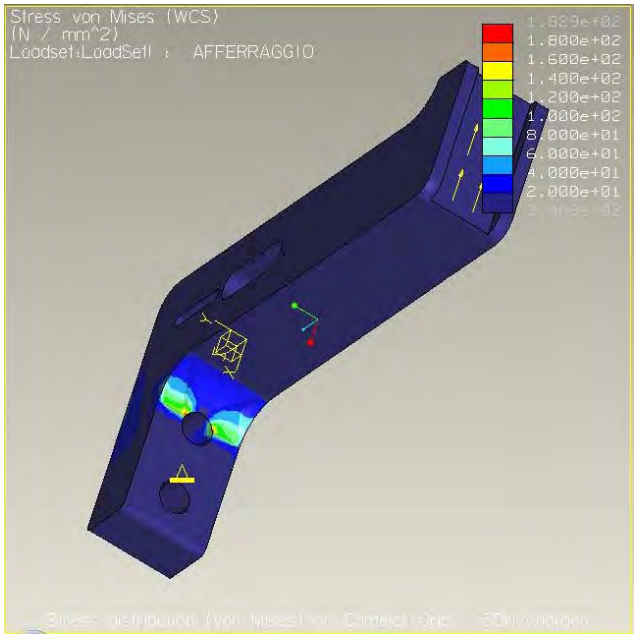


Fig. 114. Von Mises in CPDCD prototype grip under 30 N load (worst case).

A parametric study was carried out on the maximum Von Mises over the grip depending on the base thickness (Fig. 115).

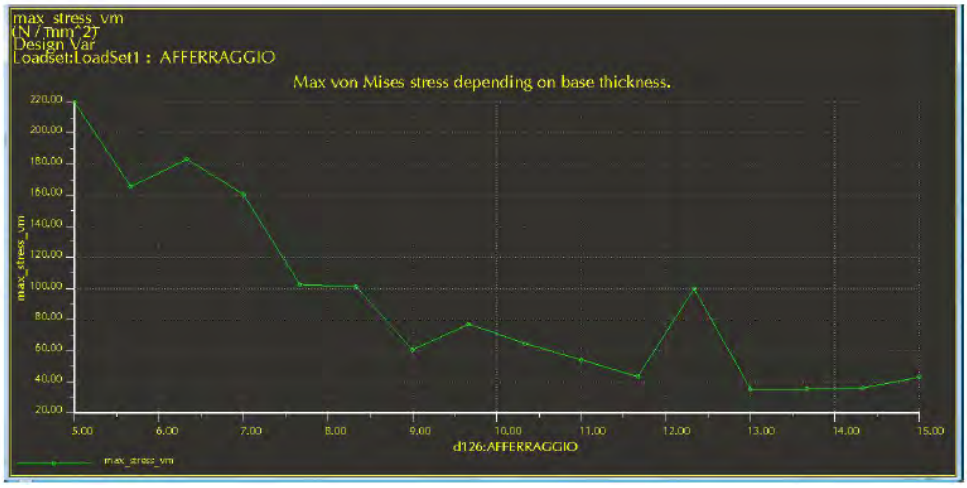


Fig. 115. Von Mises (MPa) on base thickness (mm) under 30 N load on the scaffold.

The results show that when the base is thicker than 9 mm, the local is lower than the PMMA tensile strength (72 MPa) even in the worst case, that is 30 N load on the scaffold. As a consequence, the grip deforms (Fig. 116). For these reasons, CPDCD prototype grip were re-designed (Fig. 117).

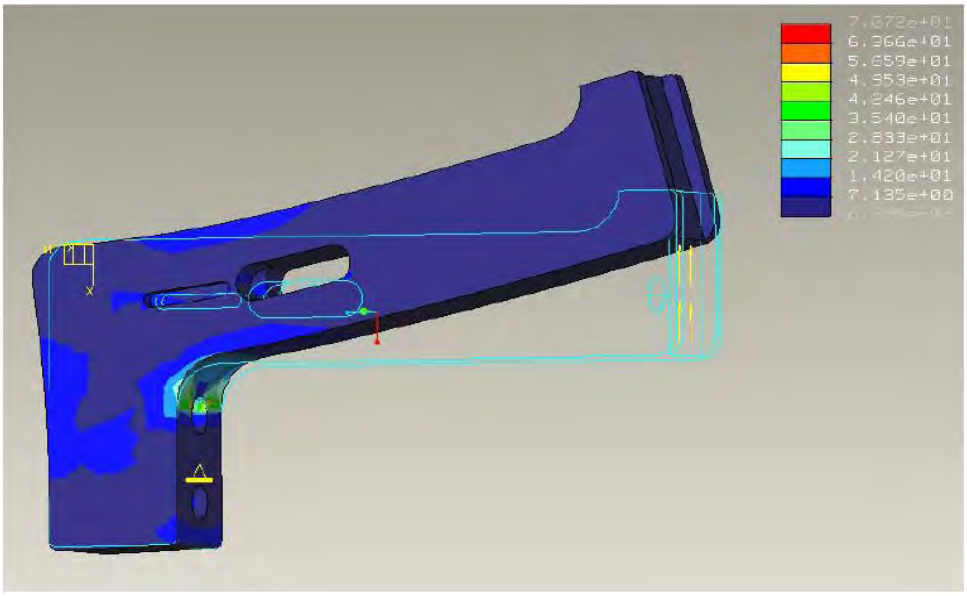


Fig. 116. Von Mises distribution (MPa) and deformation of new CPDCD prototype grip (base thickness = 9 mm) under 30 N. Maximum Von Mises is 56.59 MPa (below PMMA's tensile strength limit). Deformation enhanced via software by 200x.

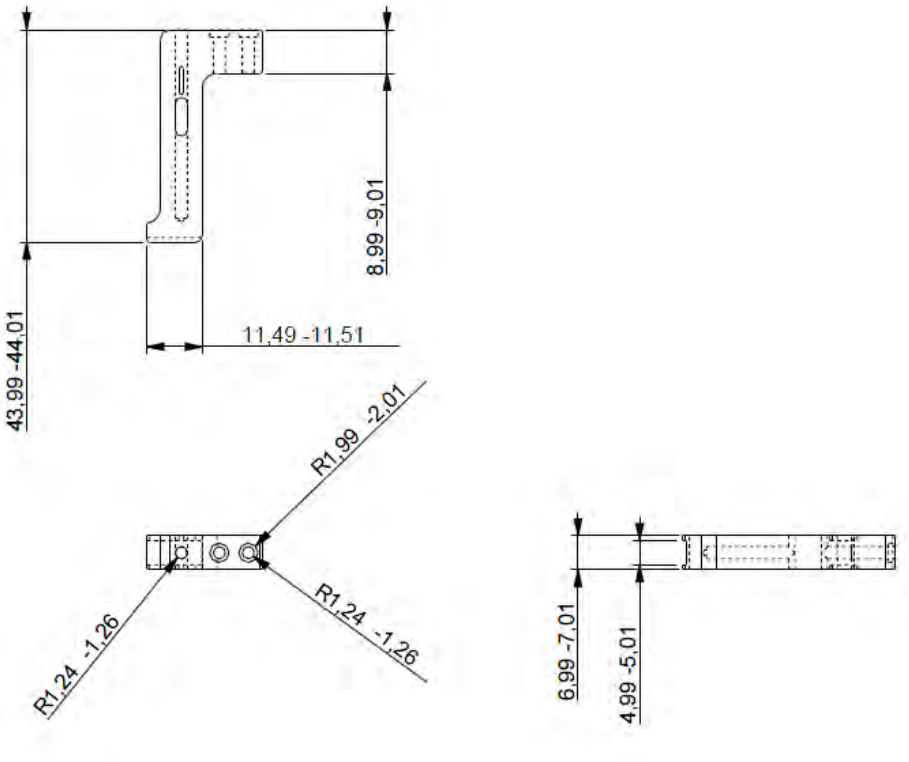


Fig. 117. Design of third review CPDCD grips (overall non refined dimensional drawing).

An alternative solution was also realized for the grip. The grip was built also using POM (polyoxymethylene) because it is autoclavable, contrary to PMMA. A FEM study was carried out also with POM. It can be noticed that POM tensile strength is still over the worst case Von Mises on the grip (Fig. 115). POM properties are reported in Table 8.

Table 8. POM properties.

Quantity	Value	Unit
Young's modulus	5200 - 5200	MPa
Shear modulus	1500 - 1500	MPa
Tensile strength	62 - 62	MPa
Elongation	12 - 12	%
Bending strength	90 - 100	MPa
Yield strength	62 - 62	MPa

Electrical stimulation subsystem

The electrical stimulation subunit (Fig. 118) is made of five AISI 316L electrodes, placed within the culture chamber, and an external voltage source. The design of this subunit has been modified with respect to Deliverable 4.1 due to reasons of ease of manufacturing and use. The electrodes, five AISI 316L bars, have been designed to be placed parallel to the constructs and in alternate position (with alternate polarity) in order to provide electrical field distributions to the cell-seeded samples. The sealed coupling between the culture chamber and the electrodes is guaranteed by silicone o-rings. An external electrical stimulator provides different stimulation outputs voltage-controlled. The implementation of the electrical stimulation subunit within the CPDCD prototype is in progress.

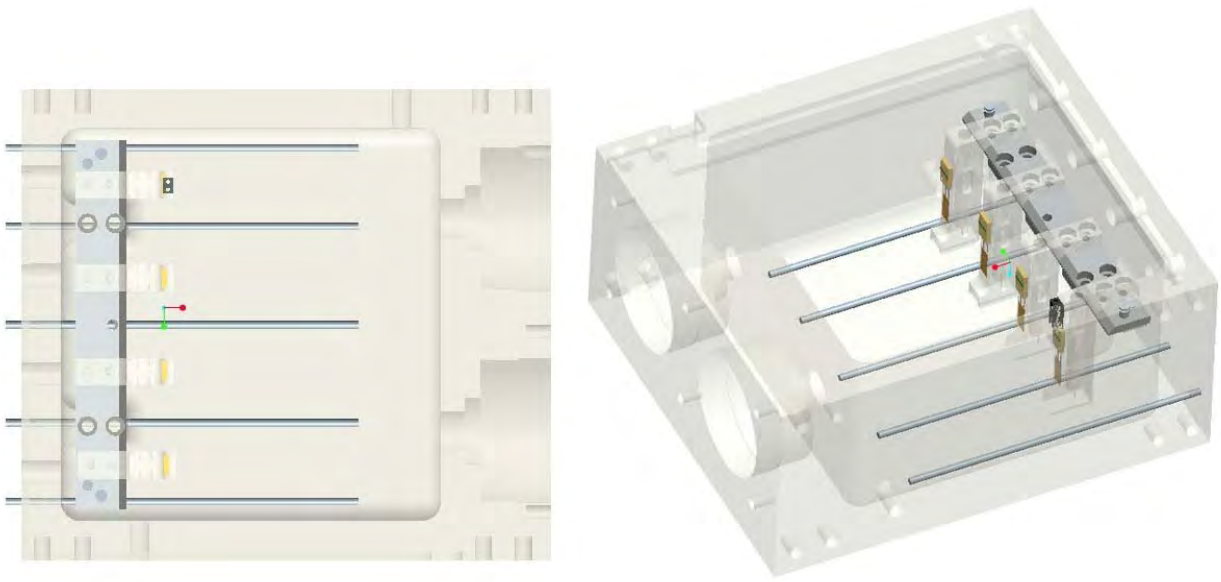


Fig. 118. Schematic drawings of the electrodes within the CPDCD prototype.

Recirculation subsystem

The recirculation subsystem (Fig. 119) is made of a media reservoir, oxygen-permeable tubes, a peristaltic pump, quick-disconnected couplings, and a waste receptacle.

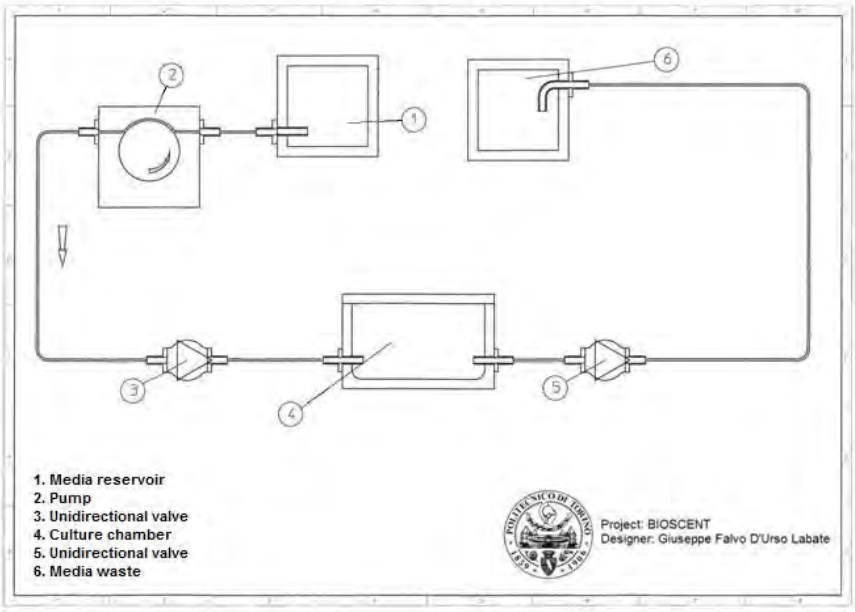


Fig. 119. Overall layout of the CPDCD prototype recirculation subsystem.

Media reservoir

The role played by the reservoir is dual: it contains sterile media for cell feeding, and it enhances mass transfer to provide oxygenated media.

Hydraulic compliance and oxygenation

The oxygenation and partially the hydraulic compliance are assured by the Masterflex BioPharm Platinum-Cured Silicone Pump Tubing (Hose barb 1/8", 3.2, Fig. 120), retaining the following features:

- Tygon 3350 silicone, platinum-cured
- Operating temperature -60 to 232°C
- Sterilization with Ethylene oxide, gamma irradiation, or autoclave for 30 min, 15 psi

Permeability properties to CO_2, H_2, O_2 and N_2 are summarized in Table 9.

Fig. 120. Masterflex BioPharm Platinum-Cured Silicone Pump Tubing.

Table 9. Permeability properties of Masterflex BioPharm Platinum-Cured Silicone Pump Tubing.

Permeability (approx.) at 25°C			
$Units: \left\{\frac{cc-mm}{sec-cm^2-cm\,Hg}\right\} x\ 10^{-10}$			
CO_2	H_2	O_2	N_2
20,132	6579	7961	2763

Pump

A peristaltic pump has been chosen (Masterflex L/S® RX-07551 – 00, Fig. 121) to assure recirculation. Pump features are summarized in the forthcoming:

- Flow rates values in the range 0.006 - 3400 ml/min
- 90/260 VAC
- rpm from 0.1 to 600
- speed control ± 0.1%
- Precision digital speed control
- Range operating temperature 0 to 40°C
- Drive dimensions 25.4 cm x 21.6 cm x 21.6 cm (10” x 8.5” x 8.5”)
- Motor (75 watts, 1/10 hp)

Fig. 121. Masterflex L/S® RX-07551 – 00 peristaltic pump.

Couplings

Cole-Parmer Quick-Disconnect couplings able to process the fluidic circulation in the system and to ensure flux unidirectionality have been chosen (Cole-Parmer SN-06364-20, Cole-Parmer SN-06361-89, Cole-Parmer SN-06364-42, Fig. 122.).

Coupling features are summarized in the forthcoming:

- Materials
  - Body: Polypropylene (PP)
  - Seal: Ethylene Propylene Rubber (EPR)
  - Springs and latches: 316 Stainless Steel
- Max vacuum 28” Hg
- Max temperature 71°C
- Max pressure at 21°C 100 psi

Technical features are reported in Table 10.

Fig. 122. Cole-Parmer Quick-Disconnect couplings SN-06364-20, SN-06361-89, SN-06364-42.

Table 10. Technical features of the Cole-Parmer Quick-Disconnect couplings SN-06364-20, SN-06361-89, SN-06364-42.

SN-06364-20	SN-06361-89	SN-06364-42
▪ Hose Barb 1/8” ID (3.2) ▪ Flow 1/8” ▪ Length 1.67”	▪ 1/8” NPT ▪ Flow 1/8”	▪ Hose Barb 1/8” ID (3.2) ▪ Flow 1/8” ▪ Length 1.05”

Waste receptacle

It is a container for temporarily storing culture wastes.

Sensing subsystem

Strain gauges

In order to evaluate the force applied to and by the construct within the culture chamber, four TML strain gauges have been purchased. These strain gauges have been stuck in the upper part of each grips (Fig. 123). Strain gauges are used to convert a displacement (strain) into an electrical signal. Their "output" (a voltage signal) is actually generated by a change in their resistance, due to a deformation.

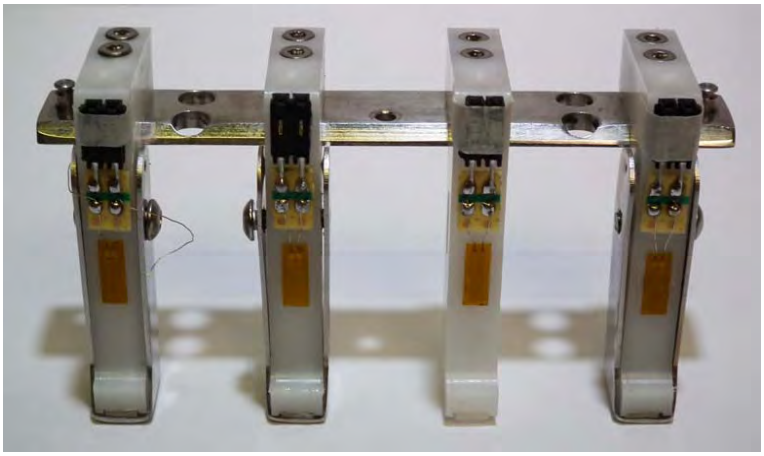


Fig. 123. Strain gauges located on the four CPDCD prototype grips.

A modification of the culture chamber (Fig. 124) has been designed for the embedding of the wires connecting the strain gauges to the control box (sensing block). This implementation is in progress.

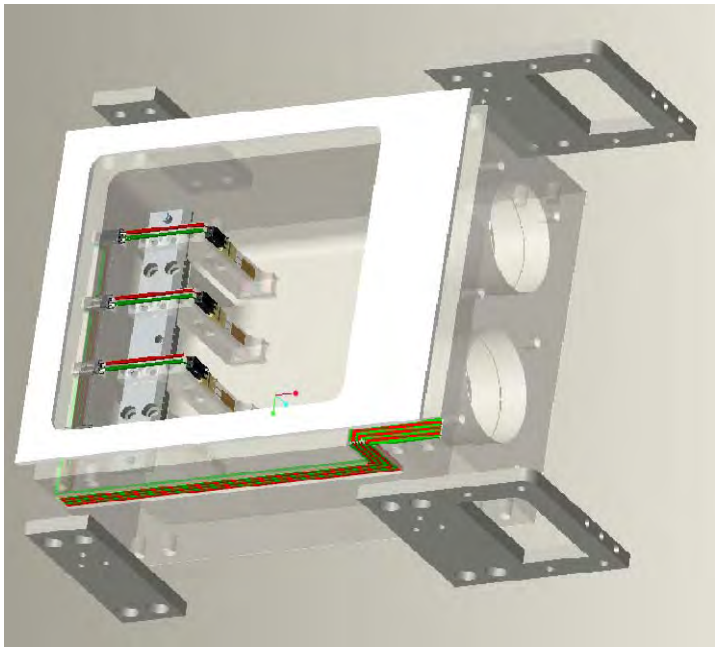


Fig. 124. Electric cables position of CPDCD prototype strain gauge subsystem.

Control subsystem

The control subsystem, placed in the control box (Fig. 125), is made of the circuitry (Fig. 126) designed and developed in order to control the CPDCD prototype stepper motor and the control box tools. The “Voltage regulator”, a LM7805, increases the voltage to 24 V in order to feed a “Ventilator” used as a cooler (working features: 24 V, 1.5 A).

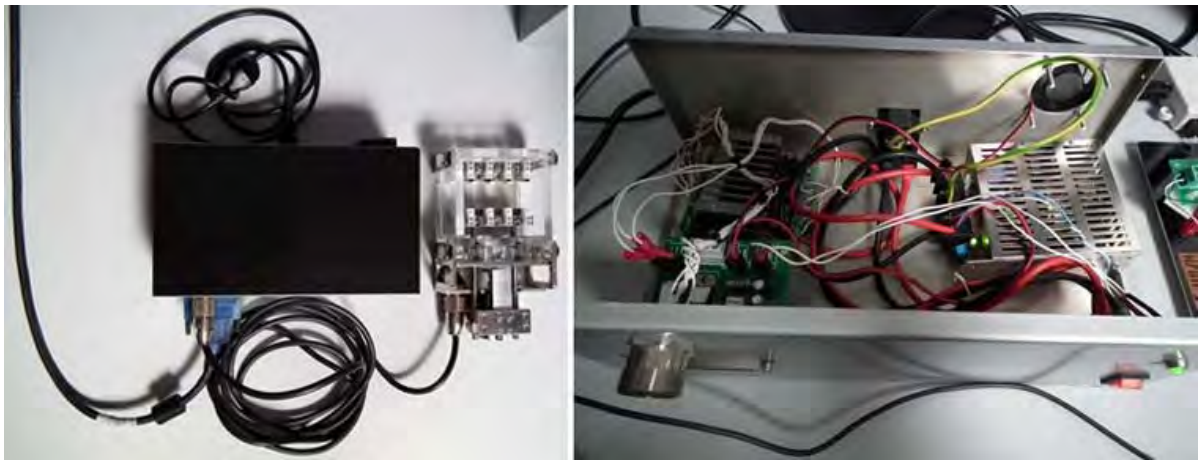


Fig. 125. CPDCD prototype set-up (left) and open control box (right).

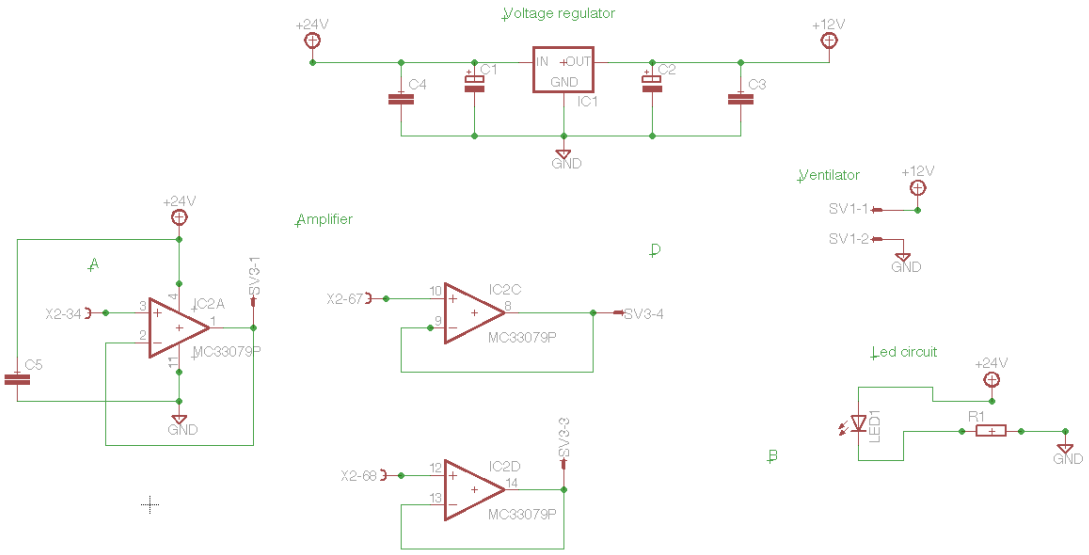


Fig. 126. Motion control circuits.

The tension supplied by the voltage regulator is also used to feed the signal amplification circuit (“Amplifier”, MC33074P) from PC to motor driver, in order to perform the movement control as set by the software. Finally, the “Led circuit” powers the LED that indicates if the control box is either on or off.

A specific circuitry (Fig. 127) has been developed in order to detect the strain signal coming from the culture chamber.

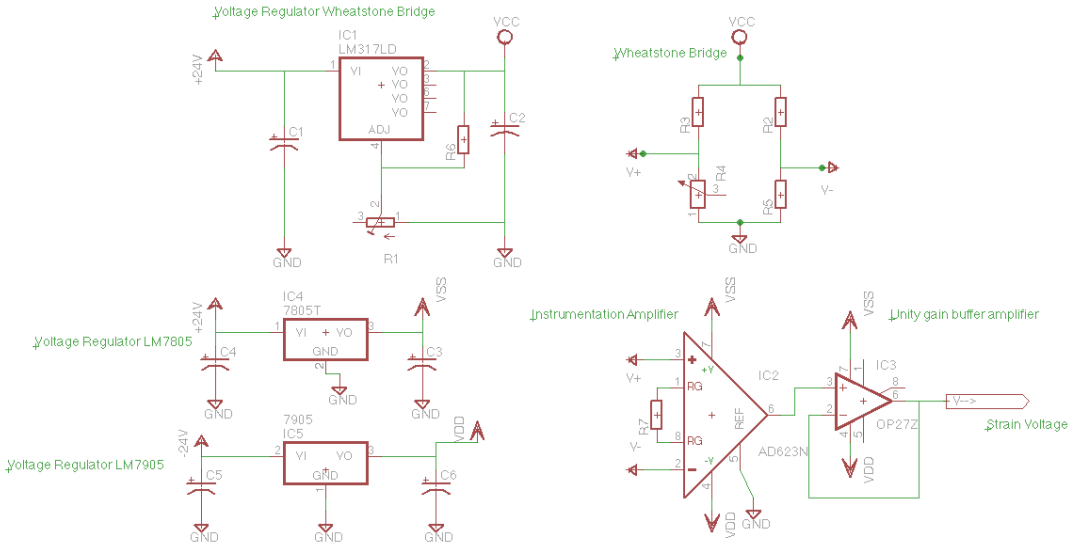


Fig. 127. Signal detection circuit.

It is based on three voltage regulators for different specifications. Two regulators, constituting the power stage, are used as instrument and buffer amplifier (LM7805 and LM7905); the third is devoted to control the voltage of the Wheatstone bridge that reveals the strain gauge resistance variation. The instrumentation amplifier is an AD623, whereas an OP27Z is used for the buffer. The output of the buffer is connected to a DAQ reading the data and sending them to PC. The Wheatstone bridge is built using precision resistors (120 Ω, 0.1% tolerance), whose voltage across (V+ and V-) gives the strain gauge resistance variation (R4 in Fig. 127) which is then amplified by an AD623.

A five points calibration procedure (Fig. 128) was performed for voltage-to-force signal conversion. Five different weights ranging from 20 to 500 g were used (20 g, 50 g, 100 g, 200 g, 500 g), and for each weight the voltage signal of the strain gauge was acquired. The test was repeated three times for each weight. For each test and for each weight, the mean value of the output voltage over the 1000 values was calculated. Finally, for each weight, the mean value of the mean output voltage was calculated over the three tests. The result is the calibration curve showed in Fig. 129. We obtained a voltage-force relationship which can be satisfactorily considered linear (R^2 = 0.98).

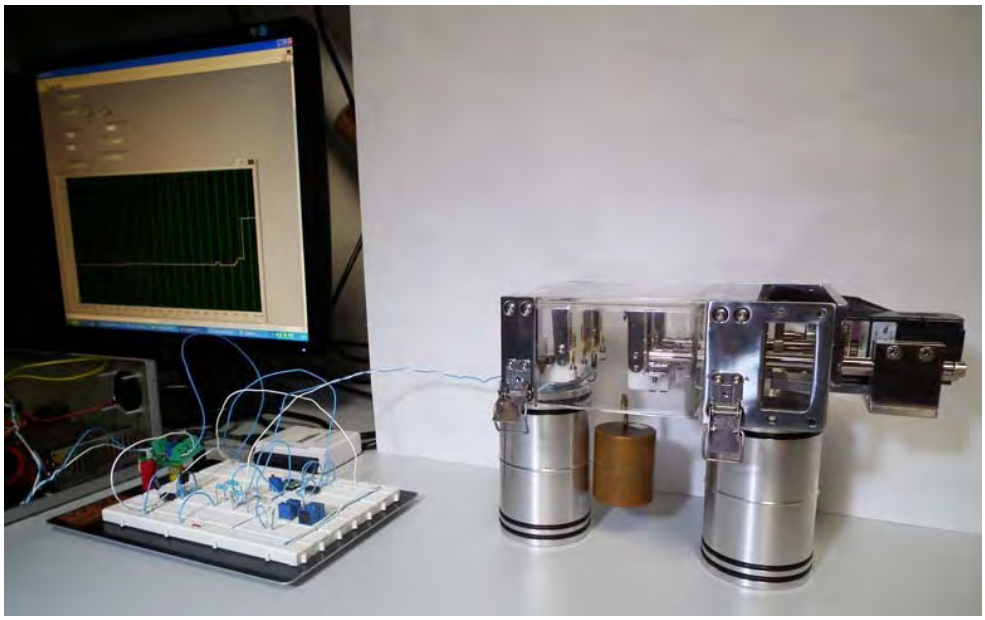


Fig. 128. CPDCD prototype calibration set-up.

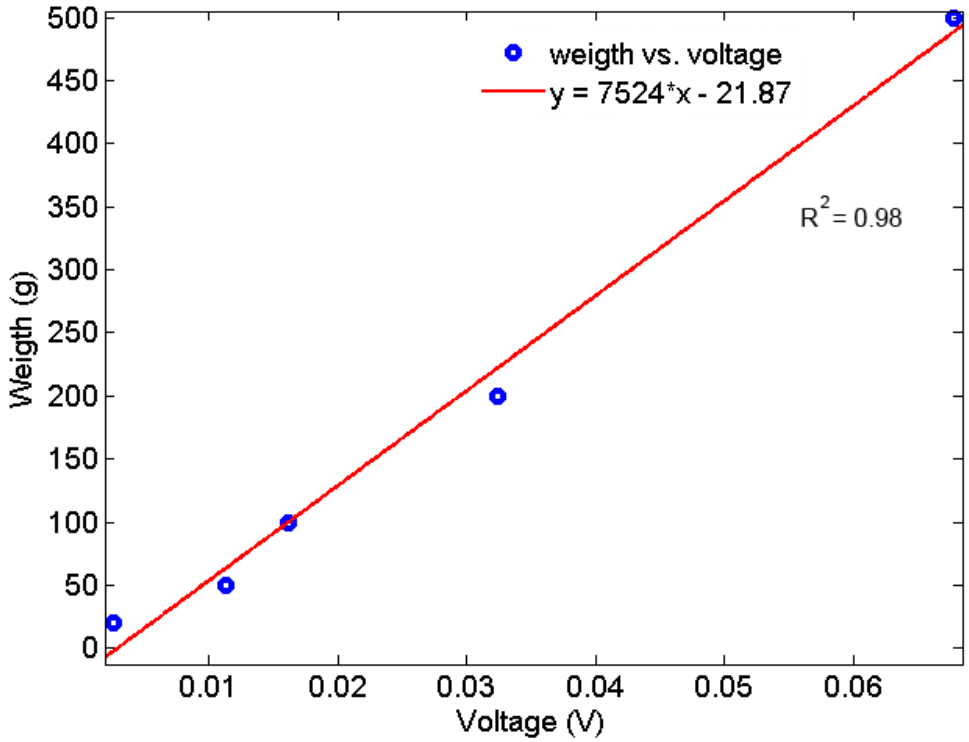


Fig. 129. CPDCD prototype calibration curve.

Software

LabView© platform (Labview 8.6 version) has been successfully used to realize user-friendly interface and modular software. The latter has been considered the main requirement. A modular software is easy to modify in any moment and by any programmer. The project has been started by creating a subunit to control the single axis stepper motor. A motion pattern has been generated using the Motion Assistant toolkit (provided by National Instruments). The pattern which has been set is a trapezoidal trajectory. A subunit dedicated to initialize the stepper motor as the sw runs has been added. This operation is compulsory before motion execution. Later, a sensing system has been designed. A further block has been added to control real-time strain (and then force) measurement by software control panel. This block is responsible for data acquisition from strain gauges.

The software is made of four blocks (Fig. 130): start, stepper motor initializing, motion control block and strain sensing block. Every software upgrade is easy-to-implement. The first block,named “Software start”, allows the user to start operations at any time by clicking on the on/off toggle switch of the control panel. Then, stepper motor is initialized (“stepper motor initializing”); this is essential for a proper functioning of the stepper motor.

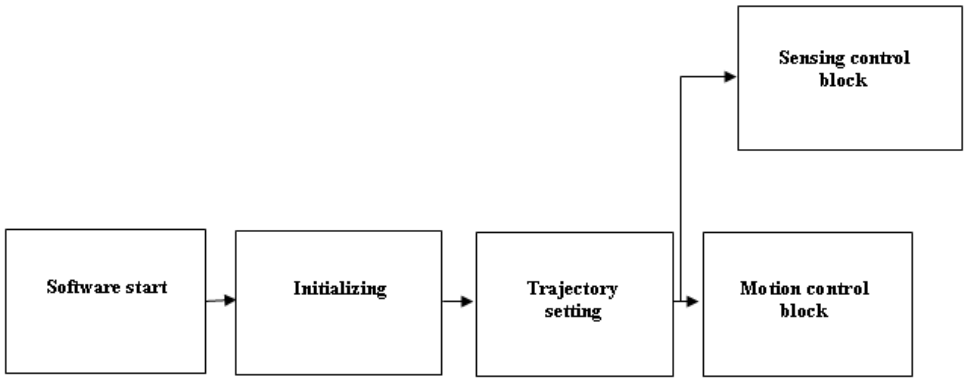


Fig. 130. Schematic of software architecture.

A third block, the “Trajectory setting”, is created to configure trajectory parameters (type, acceleration, deceleration, velocity) of the motor unit. This block allows the user to set acceleration, deceleration, velocity required for the application. The block named “Motion control block” is responsible for motion pattern control. Within this block, a control has been implemented to halt the execution either by clicking the stop button or by setting the duration of the experiment in the control panel (upper left). The block “Sensing control block” is responsible for real time signal acquisition and display. At present, only strain signal is available, but this block has been designed to be modular and ready to be used for pH, T, pO_2 signals acquisition and display, once other sensors will be installed within the culture chamber,. Software design is intended to be easily to upgrade. Motion and sensing block are independent and can work separately. The control panel (Fig. 131) allows the user to start the VI execution by clicking on the “START STIMULATION” toggle switch. User can then set stimulation AMPLITUDE (mm) and overall stimulation TIME (s). The “START SAMPLING” toggle allows the user to start and stop strain signal acquisition. The “STOP” button stops any running stimulation. Other toggles and buttons will be described in detail in an user manual.

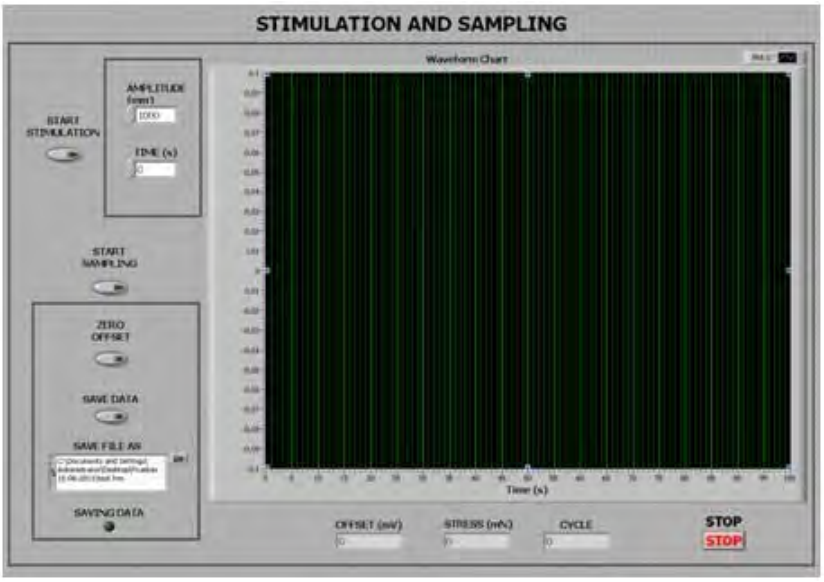


Fig. 131. Control panel divided into 2 main sections, motion and sensing.

The motion block is responsible for actuator movement pattern. In order to provide the scaffolds within the culture chamber a cyclic tensile loading, a periodic type actuator's movement has been selected. After clicking the "START STIMULATION" toggle switch on the Panel, the actuator moves periodically with a certain amplitude defined by the user. This is possible thanks to a sub-block which controls linear movements. The "AMPLITUDE (mm)" parameter, set by user, is converted into motor steps to be imposed to the motor unit. This parameter can be set from 0 to 5 mm. The sensing block is responsible for strain signal acquisition and is built by using a DAQ assistant block. A low pass filter has been added to damp DAQ noise.

Table 11. DAQ assistant block for the sensing block.

DAQ assistant input	Value
Acquisition modality	Continuous
Sample frequency	30000 Hz
Samples	10000

The software for both control and acquisition was designed to be highly user-friendly. It allows the user to easily perform three types of control over the CPDCD prototype:

- Mechanical stimulation only;
- Real time sensing only;
- Mechanical stimulation and real time sensing.

These functioning modalities are suitable for a lot of experiments aimed at investigated the environment within the culture cumber during cells stimulation or not. As up to four different engineered constructs can be hosted at the same time, this easy-to-use process control software can provide the user with several quantitative data.

Construction details

Set-up components

An overview of the CPDCD prototype set-up components is presented in Fig. 132.

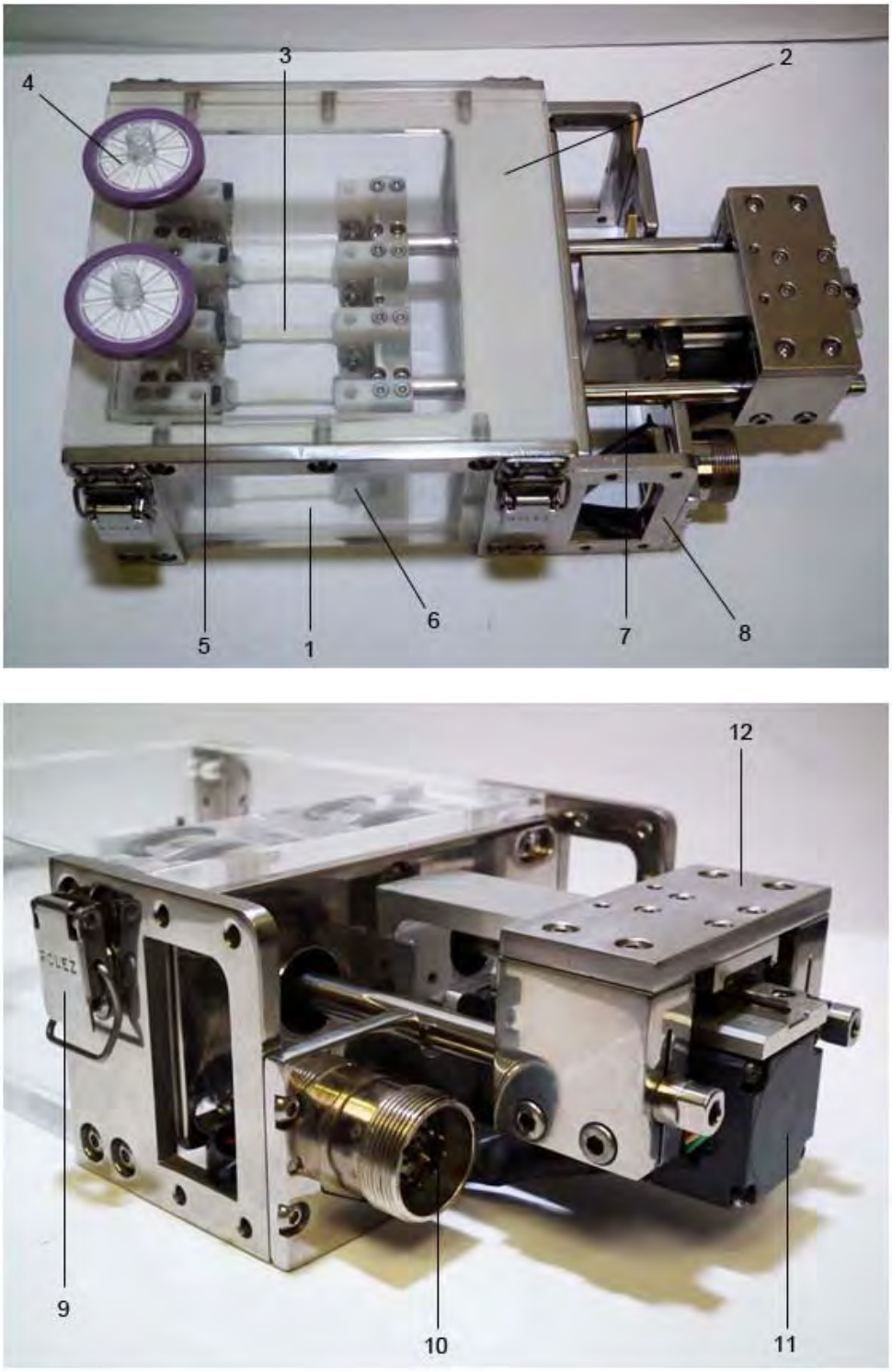


Fig. 132. CPDCD prototype set-up components: 1. Culture chamber, 2. Lid, 3. Patch sample 4. Filter, 5. Strain gauge, 6. Grip + clamp, 7. Shaft, 8. Frame, 9. Latch, 10. Cable connector, 11. Motor, 12. Shaft holder.

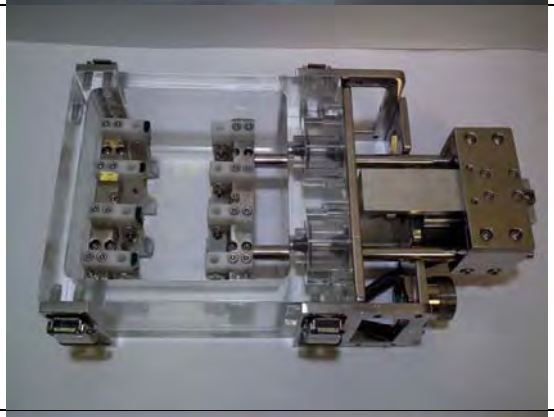
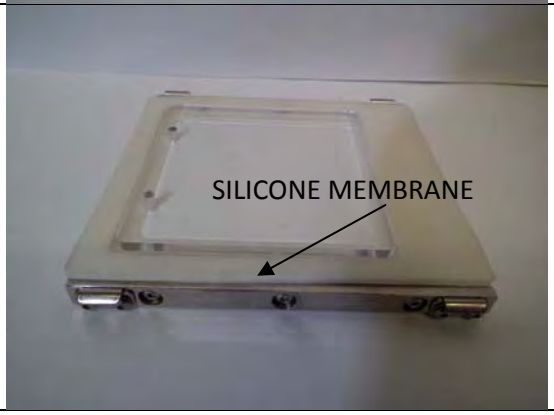
The following components can be observed:

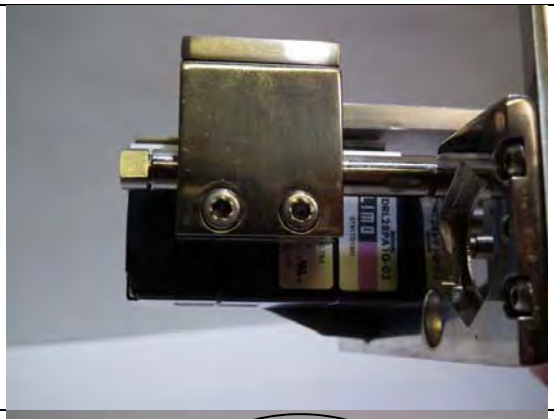
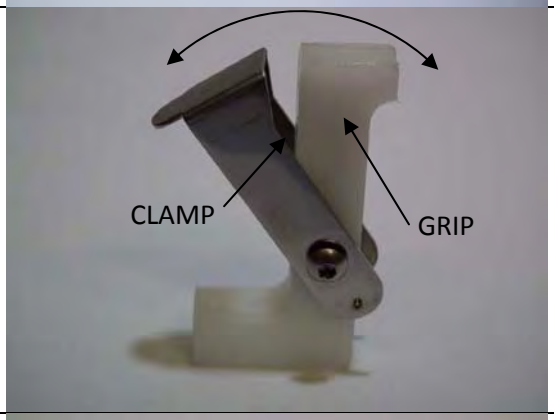
1. Culture chamber;
2. Lid;

3. Patch sample;
4. Filter;
5. Strain gauge;
6. Grip + clamp;
7. Shaft;
8. Frame;
9. Latch;
10. Cable connector;
11. Motor;
12. Shaft holder.

In Table 12, the CPDCD prototype construction details are showed and described.

Table 12. CPDCD prototype construction details.

Construction details	Description
	CPDCD PROTOTYPE SET UP The bioreactor is composed of: - Culture chamber - Lid - Mechanical stimulation subsystem (motor, grips/clamps) - Sensing subsystem (strain gauges) - Auxiliary crossbars - Auxiliary holder
	CULTURE CHAMBER Material: PMMA + AISI 316L Steel
	LID Material: Silicone Membrane + PMMA + AISI 316L Steel

	MOTOR The mechanical stimulation subsystem is composed of a 5 phase stepping motor driving two drive shafts screwed onto a crossbar that fixes four mobile grips for the samples.
	GRIP + CLAMP Material: POM + AISI 316L Steel Thanks to a spring device, the clamp can be moved according to the arrow direction in order to insert and fix the patch sample.
	STRAIN GAUGE It provides a convenient way to convert a displacement (strain) into an electrical signal. Four TML strain gauges have been purchased and stuck in the upper part of each grips.
	AUXILIARY CROSSBARS Material: AISI 316L Steel Two auxiliary crossbars are used for the patch mounting procedure.

	AUXILIARY HOLDER Material: PTFE It helps the patch mounting procedure on the grips.

Assembling procedure

The grasping system and the crossbars will be delivered fully assembled (Fig. 133).

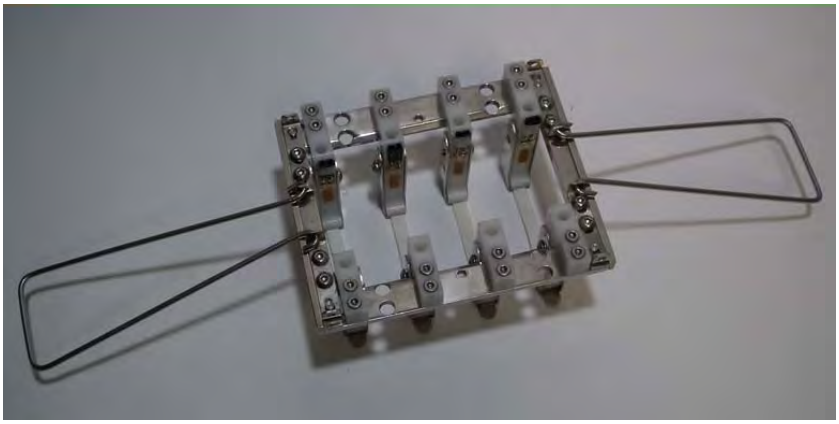


Fig. 133. CPDCD prototype grip subsystem assembling.

In Table 13, the CPDCD prototype step-by-step assembling procedure is showed and described.

Assemble the grips+clamps/crossbars using the auxiliary crossbars by lifting the spring device on the auxiliary crossbar and clamping it on the crossbar pin in order to obtain the configuration in step number 2 (Table 13).

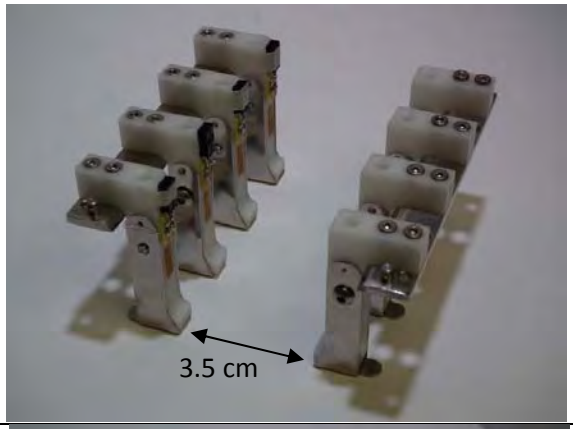
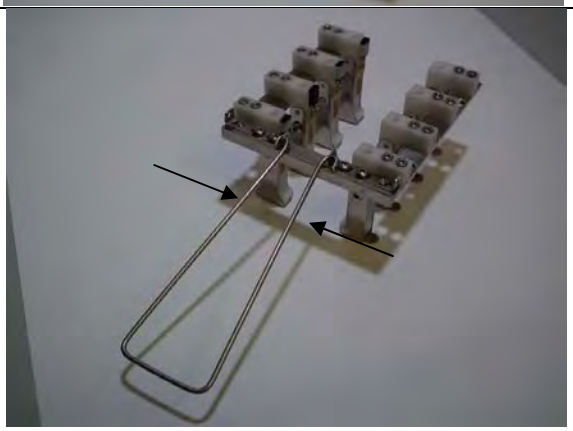
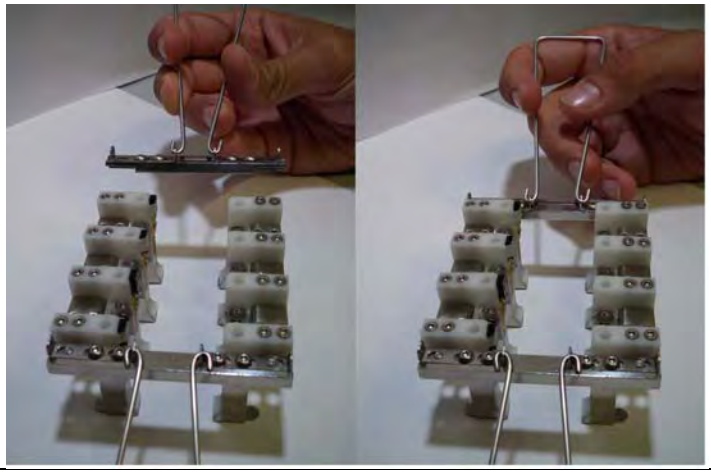
Sterilize the grips+clamps/crossbars system and the auxiliary holder in autoclave at 121°C, while the CPDCD prototype must be sterlized with Ethylene Oxide (EtO), due to the presence of PMMA and the motor.

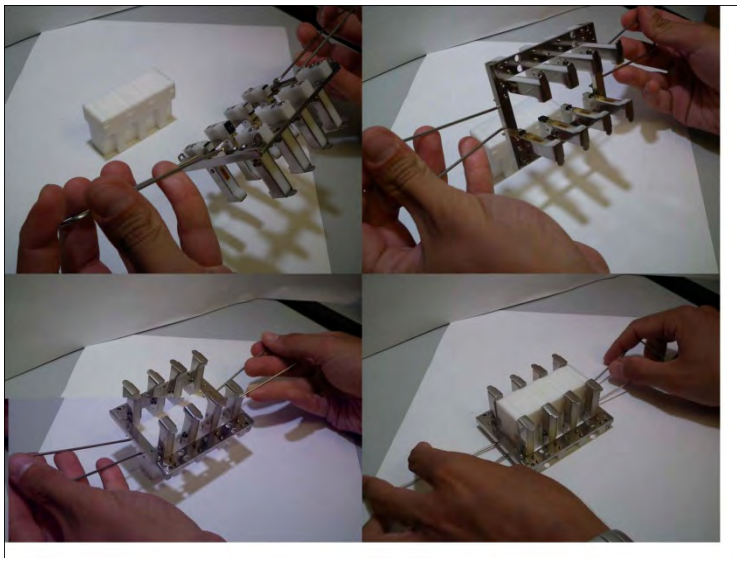
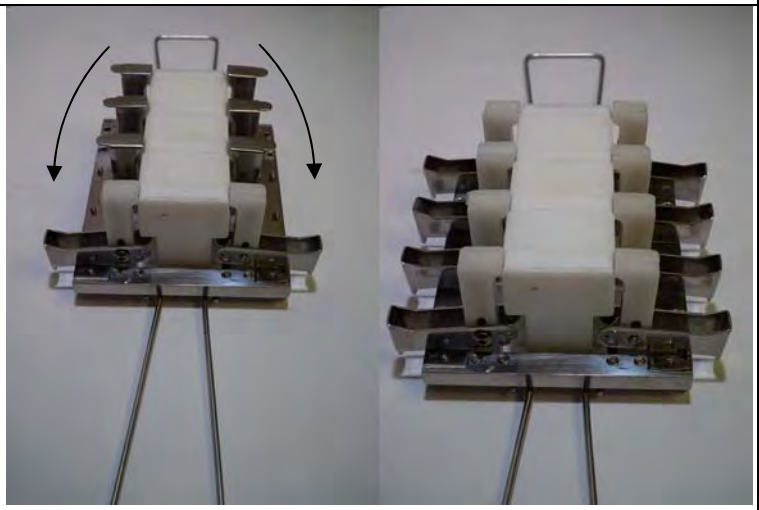
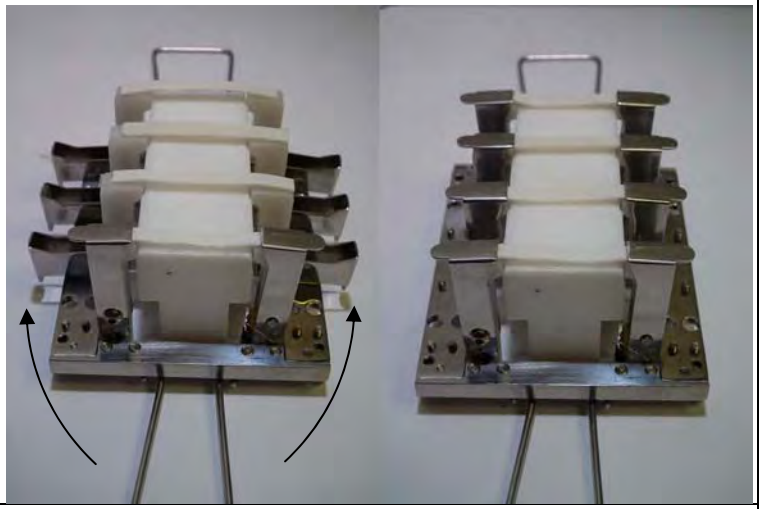
After sterilization and under hood

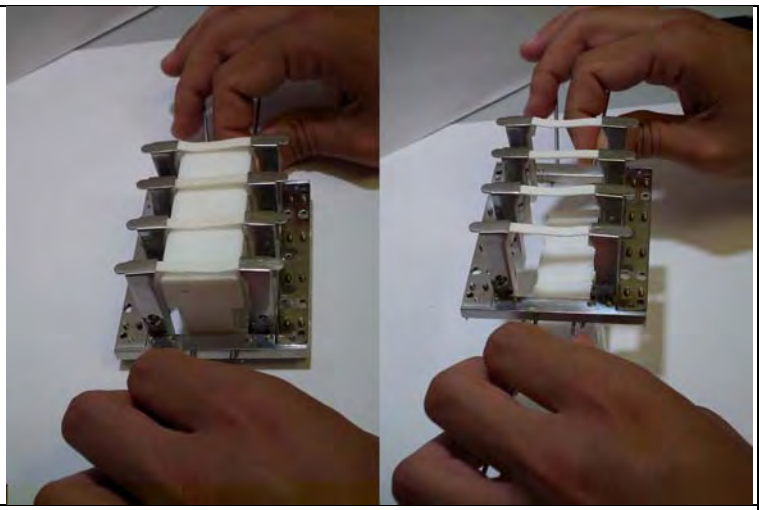
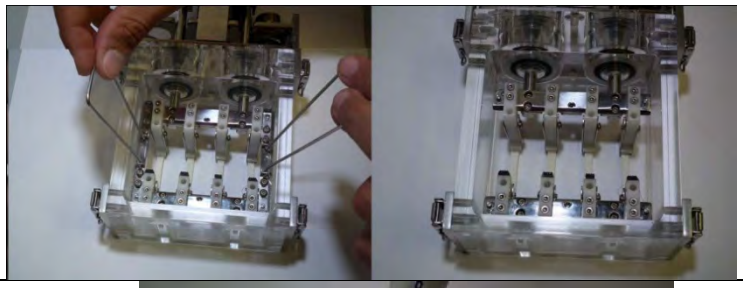
Start from step number 3 of Table 13 and proceed with the following steps.

Table 13. CPDCD prototype assembling procedure.

Assembling procedure step	Description

	STEP 1 Place the two systems (grip + clamp) in parallel at a distance of approximately 3.5 cm.
 	STEP 2 Use the two crossbars to keep the systems (grip + clamp) together. The crossbar has a spring device. Pressing the crossbar according to the arrow direction, it is possible to fix the crossbar on the system.

	STEP 3 Rotate the grip+clamp/crossbar block and insert the holder.
	STEP 4 According to the arrows direction, open the grips to allow the insertion of the patch samples.
	STEP 5 Place the four patches (5 cm x 0.5 cm) on their seats and clamp them using each couple of counter-lateral clamps.
	STEP 6 Lift the grip+clamp/crossbar block carrying the patch samples.

	
	STEP 7 Insert the patch samples in the culture chamber and remove the auxiliary crossbars using the spring device.
	STEP 8 Seed the cells on the patch samples and insert the culture medium.
	STEP 9 Put and fix the lid on the culture chamber.

Hydrogel Dynamic Culture Device (HDCD) Prototype

The Hydrogel Dynamic Culture Device (HDCD) prototype was designed to obtain a suitable biochemical and hydrodynamic environment for testing viability, growth and differentiation of cells cultured on hydrogel microsphere scaffolds developed in BIOSCENT project.

Technical requirements for HDCD

Using the information gathered by the URD (ANNEX I), technical requirements of the device were assessed as follows.

Cell type
Cell lines the CPDCD grows:

- Human Umbilical endothelial cells (HUVECs) (as a lining for antithrombogenic properties)
- Human smooth muscle cells (SMCs)
- Bone marrow mesenchymal stem cells (MSCs) (as a SMCs source if appropriated differentiation protocols are defined)
- Mouse smooth muscle cells
- Mouse/rat Bone marrow MECs (ss a SMCs source if appropriated differentiation protocols are defined)

Stimulation and perfusion
BIOSCENT hydrogels requires rotating environment.

A static culture medium is needed during seeding phase, then rotation is required while culturing.

Performance requirements

Stimulation and perfusion	
Parameter	**Value (or range)**
Rotation speed [s^{-1}]	0.5 - 5
Perfusion flow [mm^3/s]	5.3 - 25
Perfusion pressure [mmHg]	Environment pressure

Measurement	
Parameter	**Value (or range)**
Temperature [°C]	37
Dissolved CO_2 [mmHg]	48 - 55
Dissolved O_2 [mmHg]	115 - 130
Glucose consumption rate [g/l/day]	2.216±0.02
Lactate consumption rate [g/l/day]	1.436±0.12
Glutamine rate [mM]	2 – 4
pH	7.21 – 7.33

Design requirements	
Feature	**Requirement**
Priming volume [ml]	30

Architectural design

The HDCD prototype is characterized by peculiar geometric features which assure the possibility for buoyant vortices to be generated. The establishment of this fluid dynamic regime within the device allows (i) to maintain specimen of different dimensions (i.e., cells, carriers etc.) in suspension conditions, and (ii) to guarantee a suitable oxygen transport by assuring laminar flow conditions without using electromechanical rotating systems.

Key constitutive elements of the HDCD prototype are (Fig. 134):

- a transparent, sealable and sterile culture chamber where cells, seeded on hydrogel microspheres, and culture medium are housed during the experiments;
- a perfusion system, constituted by a media reservoir, a rotary pump, and oxygen permeable tubes (oxygenation module), that is designed to produce the rotating microgravity environment needed to culture cell-seeded hydrogels properly.

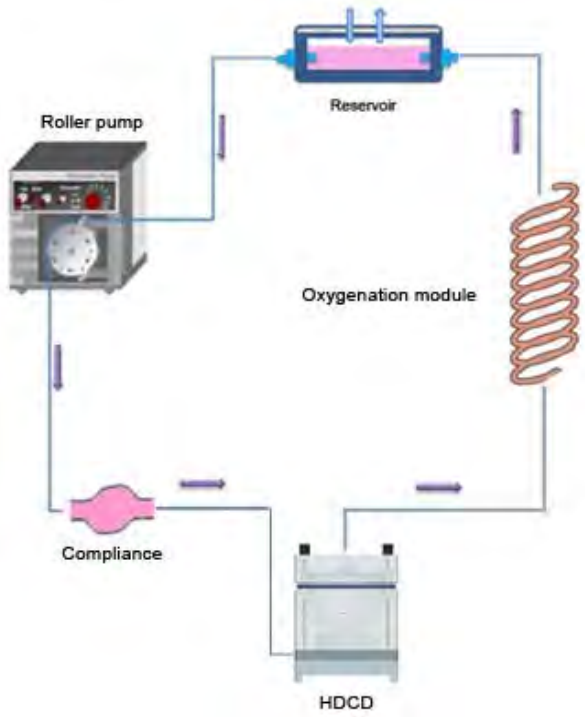


Fig. 134. Architectural design of HDCD prototype constituted of: a medium reservoir; a peristaltic pump; a culture chamber; and oxygen permeable tubes.

The HDCD prototype was realized by satisfying the following constraints and requirements:

- cytocompatibility of all the materials in contact with culture medium;
- corrosion-resistance of the materials in contact with culture medium;
- ease of sterilization and sterility maintenance;

- ease of use (assembly in sterile conditions under a laminar flow hood, medium exchange, cleaning, use for non-trained staff);
- small dimensions, suitable for positioning in a cell culture incubator;
- no medium stagnation during exchange operations.

Detailed list of components

Culture chamber

The geometry of the HDCD prototype culture chamber (Fig. 135) is internally cylindrical, as easily found in literature (see paragraph 2.2.1, Fig. 64 and Fig. 65). The top of HDCD chamber (Fig. 135) has a convergent geometry in order to minimize flow turbulence and promote velocity uniformity, avoiding bubble formation (see paragraph 2.2.1 and Fig. 67). It was designed with the final goal to establish the formation of a buoyant, stationary vortex within the culture medium: the establishment of this vortex allows (i) to maintain cultured cells in suspension and (ii) to guarantee a suitable oxygen transport within the chamber. In the HDCD prototype, the oxygenated culture medium enters from the base through a quick-disconnect coupling, moves through the check valve, pervades the culture chamber, and flows out from the top of the culture chamber through a quick-disconnect coupling. Within the culture chamber are located a filter, designed in order to prevent accidental outputs of cell-seeded hydrogels during the recirculation of the culture medium, and a check valve for guarantying the unidirectionality of the flow.

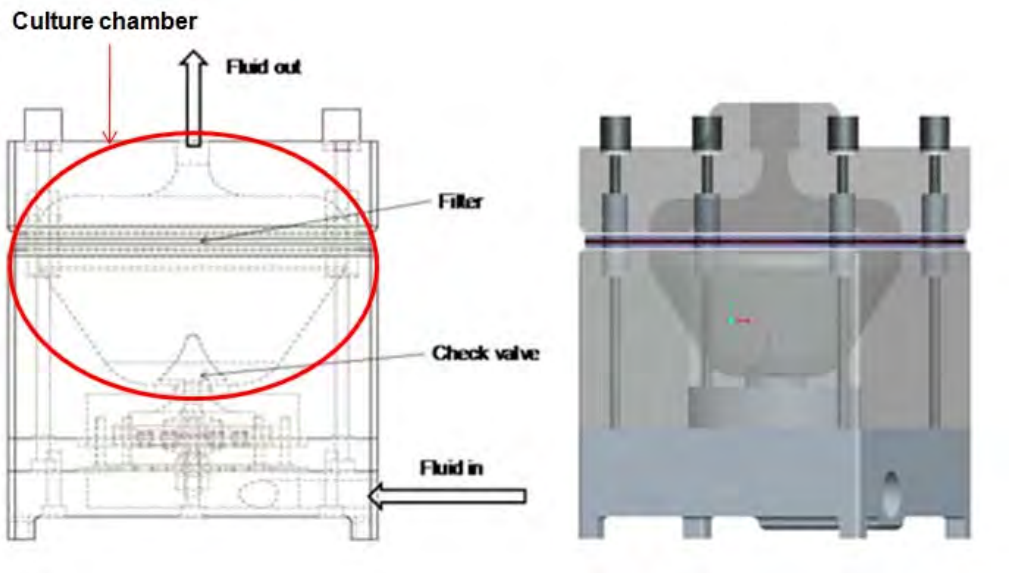


Fig. 135. HDCD prototype design.

The maintenance of adequate suspension conditions can be achieved through the attainment of a dynamic balance among the different forces acting simultaneously. The result of the balance between hydrodynamic and gravitational forces is the attainment of microgravity for cultured cells, within a dynamic environment in which the generation of cell-damaging hydrodynamic forces is minimized and the nutrient supply to the cultured cells is maximized.

In the environment of the HDCD prototype, the proper combination (i) of the fluid dynamic conditions establishing at the inlet vane of the chamber with (ii) the shape of the side walls of the chamber itself give rise to flow separation, with the consequent formation of stationary buoyant vortices and of hydrodynamic forces the resultant of which balances the gravitational force, thus avoiding the sedimentation of cultured specimen at the bottom of the culture chamber. In detail, the pull of the gravity force acting on the

suspended beads is balanced by equal and opposite hydrodynamic-induced forces. Gravitational and hydrodynamic forces, including buoyant and drag forces which result from the operating chamber in the gravitational field, combine simultaneously to produce a “suspension” condition for the immersed particles. In this way particles experience an average near zero gravitational force, thereby leading to what has been described as the “simulated microgravity” condition. Furthermore, the buoyant vortex assures proper mixing within the chamber and hence oxygen uptake to the cells.

The fluid (i.e., the culture medium) is mixed within a laminar flow environment and potentially damaging cell due to bead-fluid friction and bead-bead collisions is minimized in microgravity conditions, conversely to what has been reported for dynamic bioreactors based on the agitation/stirring mechanism, where the onset of turbulences in the fluid flow typically occurs.

These design philosophy allows to not incorporate rotating components within the device.

Check valve

The presence of a check valve (Fig. 136) ensures unidirectional motion of the culture medium within the circuit. The valve motion from the closed to the open position and vice versa is vertical and it is obtained by means of a moving silicone membrane (Fig. 137).

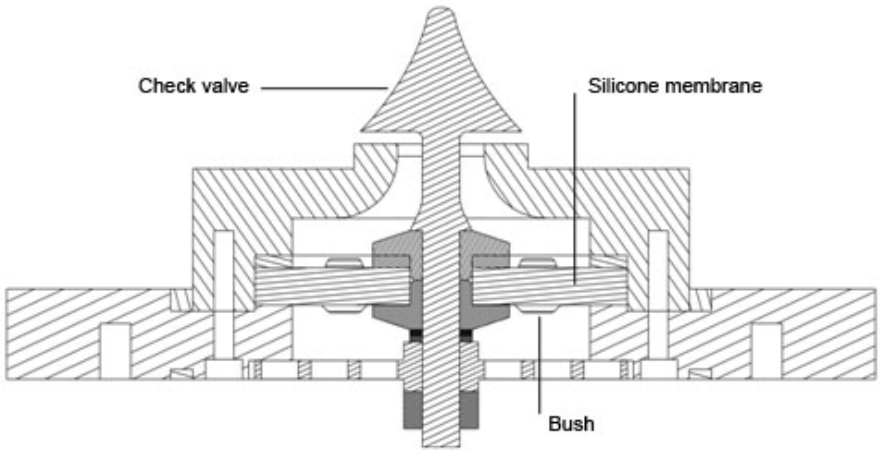


Fig. 136. HDCD prototype check valve design.

The transport of the culture medium in the device is achieved by production of over pressures in the pump chamber, with respect to the culture chamber: the medium flows only when the transvalvular pressure difference overcomes the cracking pressure of the valve. The silicon membrane is designed with six holes, each one of them housing a bushing that can be perforated or closed so that the proper operating transvalvular pressure can be modulated.

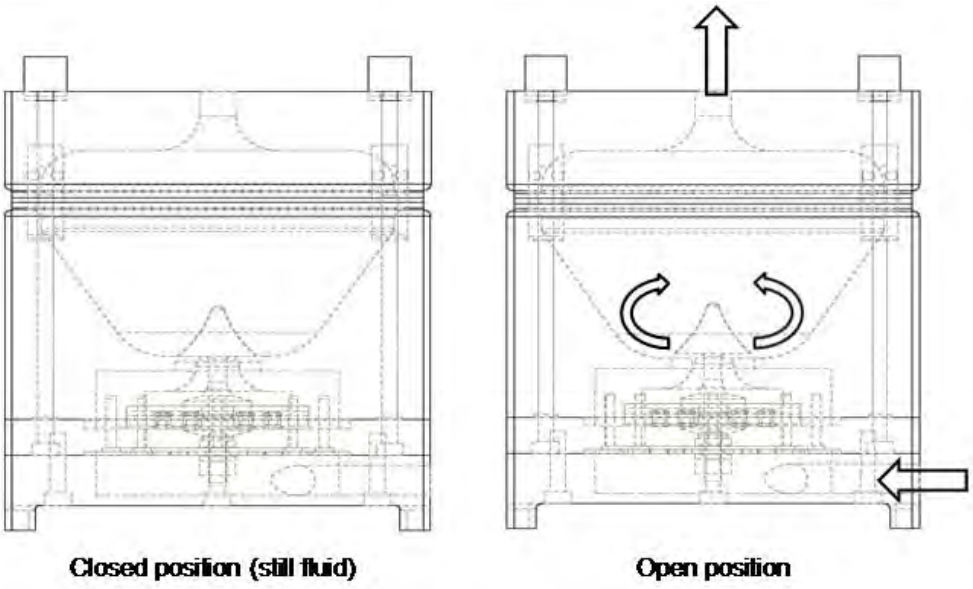


Fig. 137. HDCD prototype check valve in closed (left) and open (right) position.

Perfusion subsystem

The perfusion subsystem (Fig. 138) is composed of a media reservoir, a peristaltic pump, quick-disconnected couplings, and oxygen-permeable tubes.

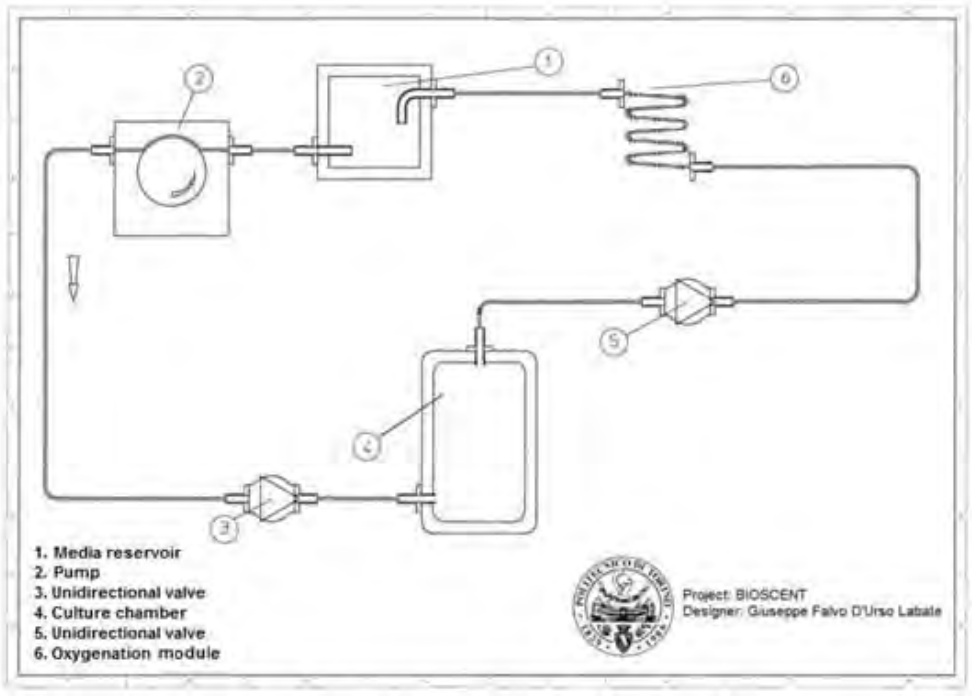


Fig. 138. Overall layout of the HDCD prototype perfusion subsystem.

The perfusion subsystem elements are the ones used for CPDCD, as previously described.

Construction details

Set-up components

An overview of the HDCD prototype set-up components is presented in Fig. 139.

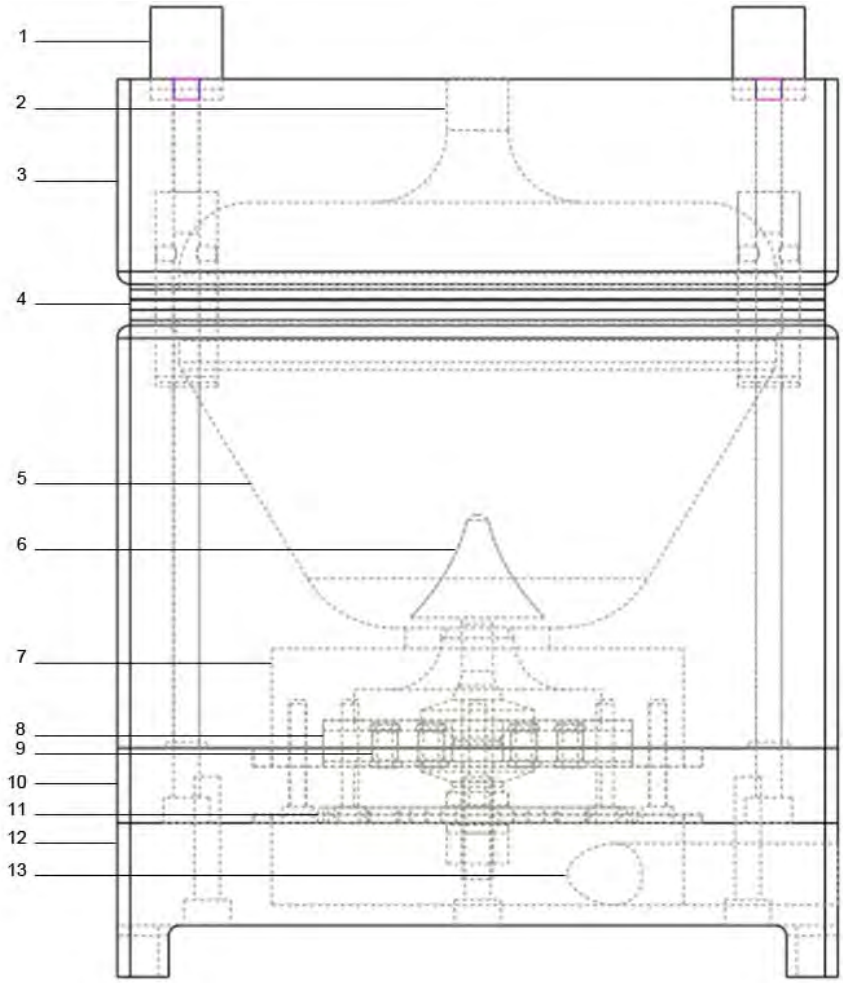


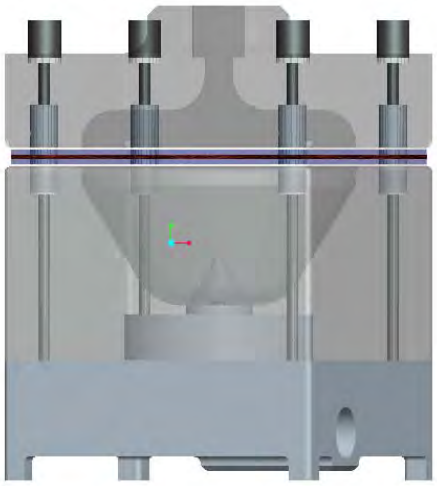
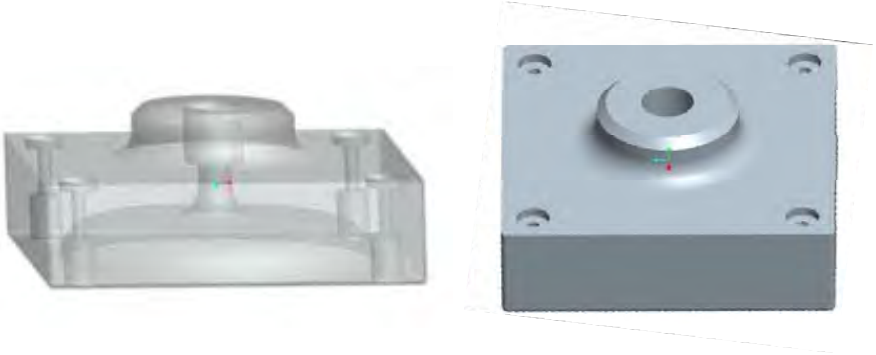
Fig. 139. HDCD prototype set-up components: 1. Thumb screw, 2. Flow outlet, 3. Lid, 4. Filter, 5. Culture chamber, 6. Check valve, 7. Membrane holder 1, 8. Membrane, 9. Bushing, 10. Membrane holder 2, 11. Perforated plate, 12. Base, 13. Flow inlet.

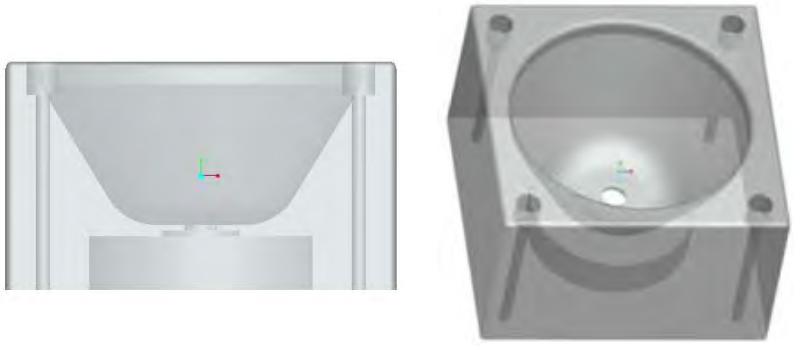
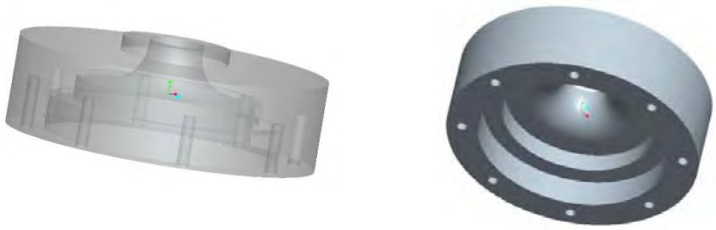
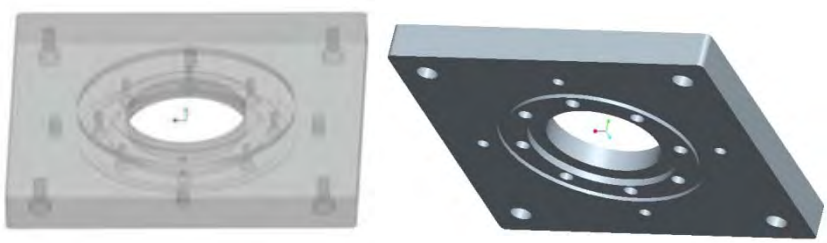
The following components can be observed:

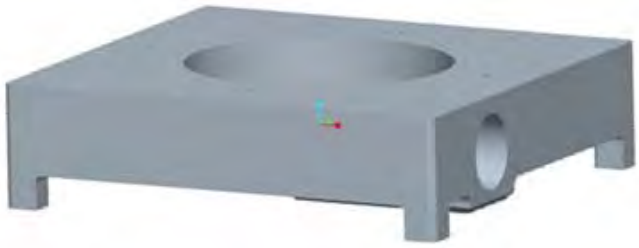
1. Thumb screw;
2. Flow outlet;
3. Lid;
4. Filter;
5. Culture chamber;
6. Check valve;
7. Membrane holder 1;
8. Membrane;
9. Bushing;
10. Membrane holder 2;
11. Perforated plate;
12. Base;
13. Flow inlet.

In Table 14, the HDCD prototype construction details are showed and described.

Table 14. HDCD prototype construction details.

Construction details	Description
	HDCD PROTOTYPE SET UP The bioreactor is composed of: - Flow outlet - Lid - Filter - Culture chamber - Check valve - Membrane - Base - Flow inlet Approximate dimensions: 70 mm x 70 mm x 94.5 mm
	THUMB SCREW Material: AISI 316L Steel Four lid closures are present on the top of the lid in order to fix it on the culture chamber.
	LID Material: Polycarbonate (PC) On the top it presents the site for the quick-disconnect coupling for the flow outlet.
	FILTER Material: AISI 316L Steel

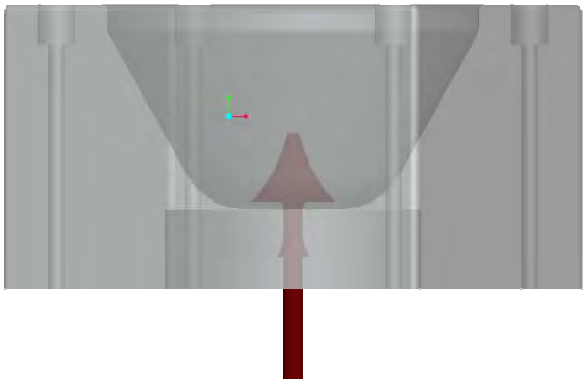
	CULTURE CHAMBER Material: Polycarbonate (PC)
	CHECK VALVE Material: AISI 316L Steel
	MEMBRANE HOLDER 1 Material: AISI 316L Steel For fixing the silicone membrane, allowing the up and down motion.
	MEMBRANE Material: Silicone It contains 6 holes in order to accommodate bushings.
	BUSHING Material: AISI 316L Steel
	MEMBRANE HOLDER 2 Material: AISI 316L Steel For fixing the silicone membrane, allowing the up and down motion.

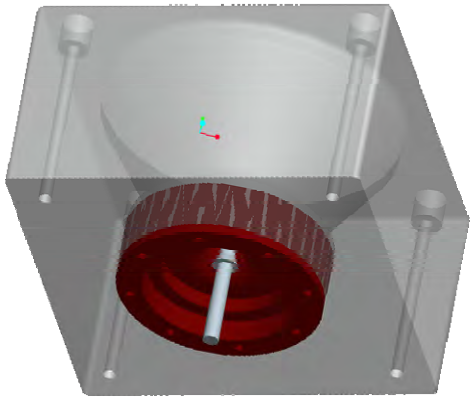
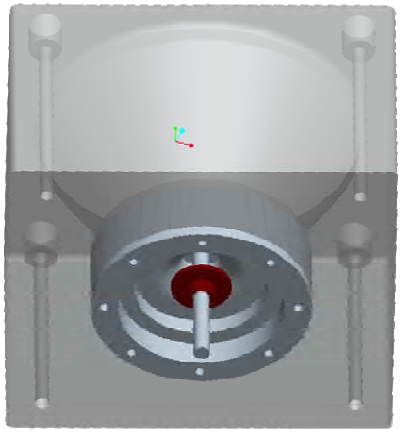
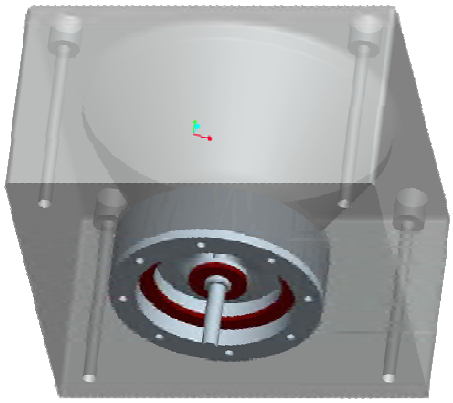
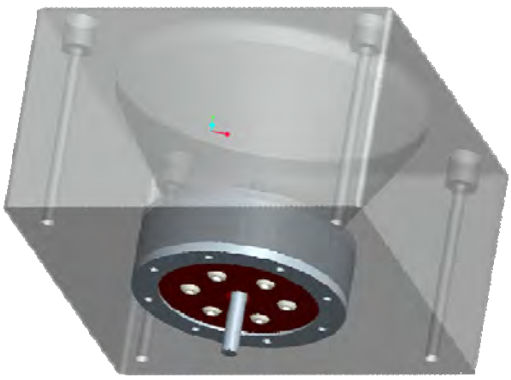
	PERFORATED PLATE Material: AISI 316L Steel Thanks to holes, it allows the passage of the fluid and it contributes to recreate the necessary pressure to lift the check valve and the membrane.
	BASE Material: AISI 316L Steel On one side it presents the site for the quick-disconnect coupling for the flow inlet.

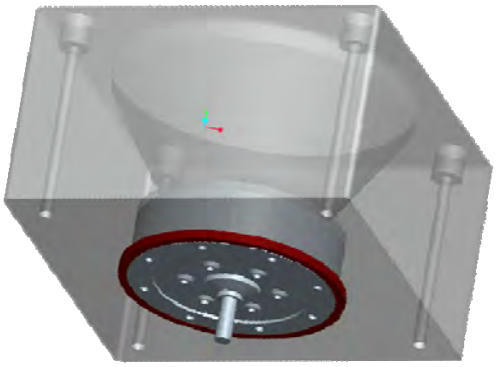
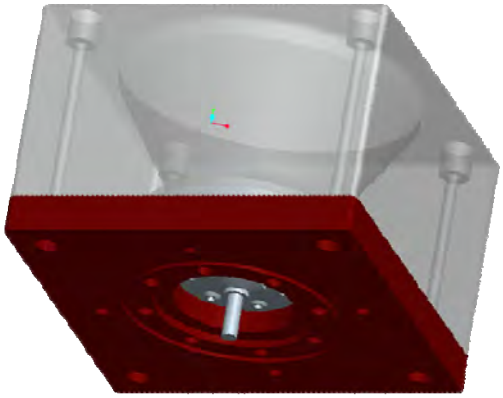
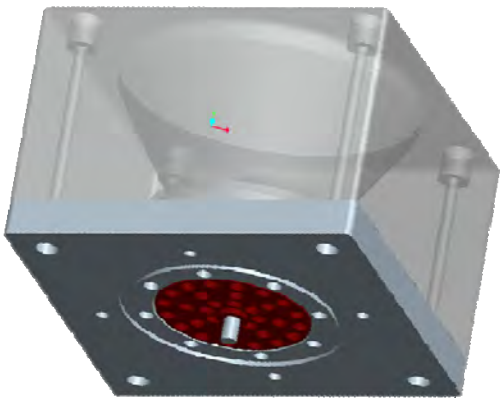
Assembling procedure

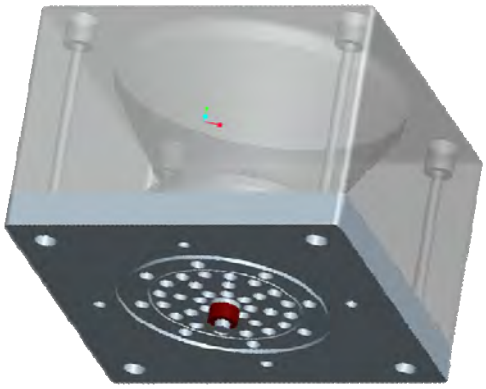
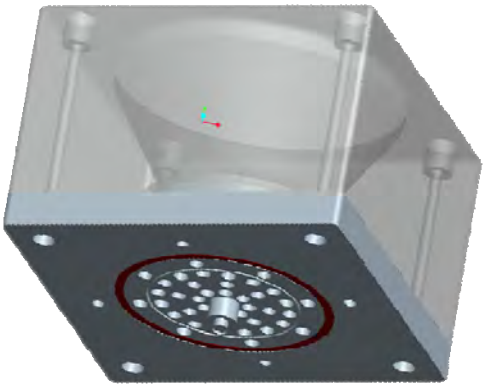
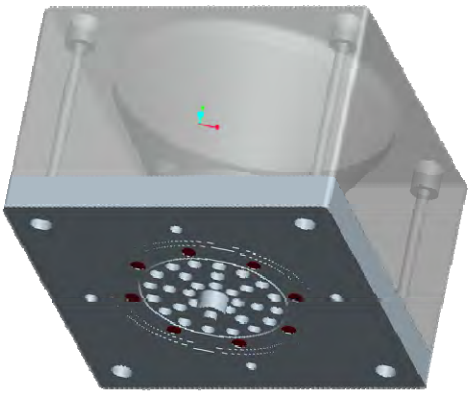
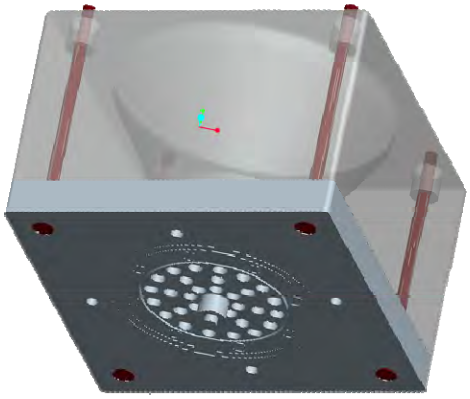
Sterilize the HDCD prototype components in autoclave at 121°C. After sterilization, the assembling procedure steps summarized in Table 15 must be followed under hood.

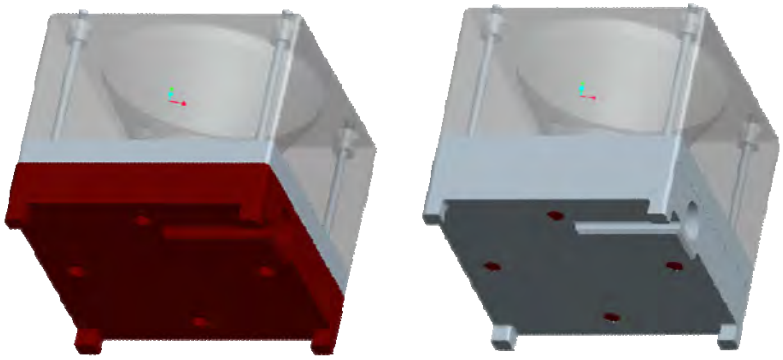
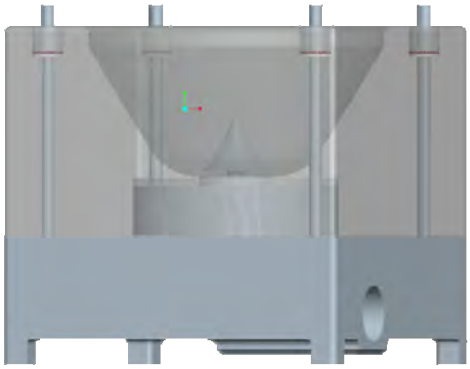
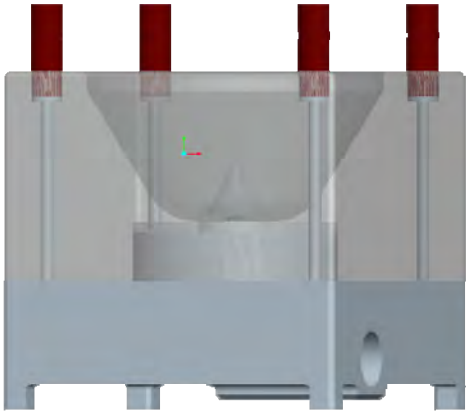
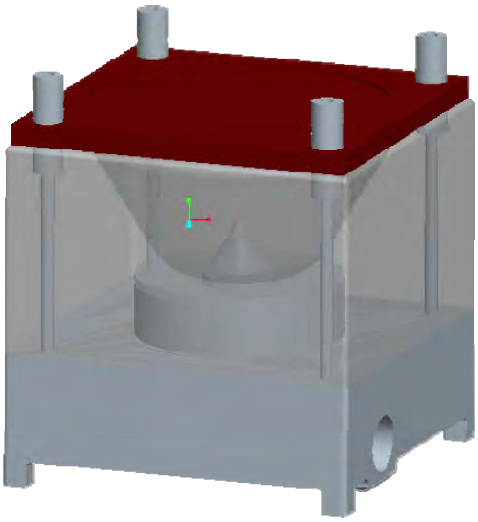
Table 15. HDCD prototype assembling procedure.

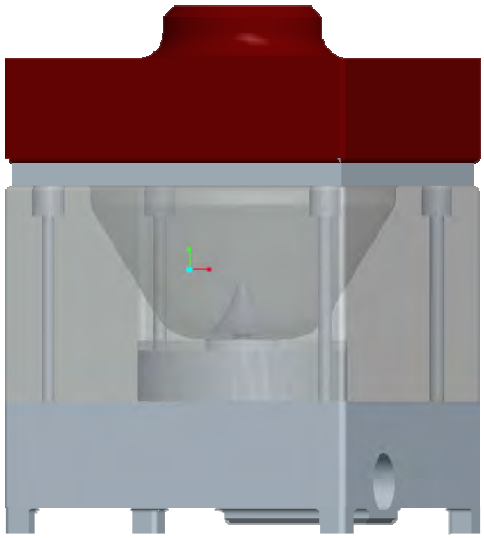
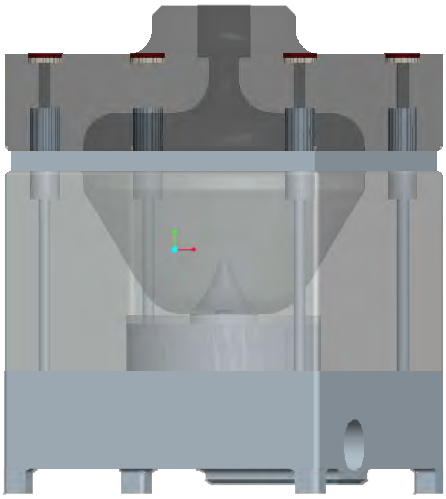
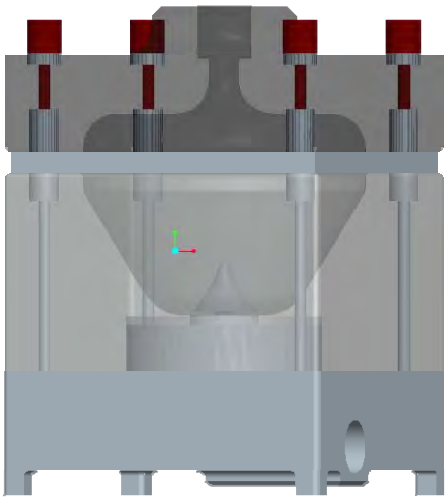
Assembling procedure step	Description
	STEP 1 Insert the check valve within the lower part of the culture chamber.

	STEP 2 Insert the membrane holder 1 on the cylindrical shaft of the check valve.
	STEP 3 Insert the AISI 316L Steel washer on the cylindrical shaft of the check valve.
	STEP 4 Insert the smaller AISI 316L Steel o-ring on the cylindrical shaft of the check valve and the larger one in contact with the membrane holder 1.
	STEP 5 Insert the silicone membrane, with the selected bushings, on the cylindrical shaft of the check valve.

	STEP 6 Insert the AISI 316L Steel washers on the cylindrical shaft of the check valve.
	STEP 7 Insert the silicone o-ring in contact with the membrane holder 1.
	STEP 8 Insert membrane holder 2 on the cylindrical shaft of the check valve.
	STEP 9 Insert perforated plate on the cylindrical shaft of the check valve.

	STEP 10 Insert the nut on the cylindrical shaft of the check valve.
	STEP 11 Insert silicone o-ring in the cavity of the membrane holder 2.
	STEP 12 Fix each component with the 1.6 MA screws.
	STEP 13 Screw 2.5 MA screws in the four corners of the membrane holder 2.

	STEP 14 Position the base and fix it with 2.5 MA screws.
	STEP 15 Insert in the four cavities of the culture chamber the silicone o-rings and the AISI 316L Steel washers.
	STEP 16 Insert in the four cavities of the culture chamber the cylinders.
	STEP 17 Insert the filter set on the four cylinders.

	STEP 18 Place the lid on the culture chamber.
	STEP 19 Insert in the four cavities of the lid the silicone o-rings and the AISI 316L Steel washers.
	STEP 20 Fix the lid with the four thumb screws.

Behaviour/operation testing of HDCD prototype components/subsystems

In order to assess the suitability of the design of the HDCD prototype culture chamber for favouring the formation of buoyant stationary vortices and for guarantying microgravity conditions within the culture medium, computational fluid dynamic simulations were carried out. The computational approach allowed to investigate the effect of the HDCD prototype design on suspension of hydrogel microspheres. In

particular, the aim of the computational simulation was to assess the homogeneity of the distribution of beads floating in simulated microgravity conditions, and to verify whether the imposed culture conditions avoid that the microspheres (1) sediment at the bottom of the chamber, (2) crush on the filter or form excessively packed aggregates, and (3) collide with the boundary walls of the chamber, thus eliciting cell damage or their rupture.

For the mesh generation (Fig. 140), the solid modeler Gambit (GAMBIT, ANSYS, Inc., US) was used. The numerical simulations were carried out using a properly customized finite volume technique-based commercial software (FLUENT, ANSYS Inc., US). Since the HDCD prototype is characterized by an axial symmetry, axisymmetric numerical model simulations were performed.

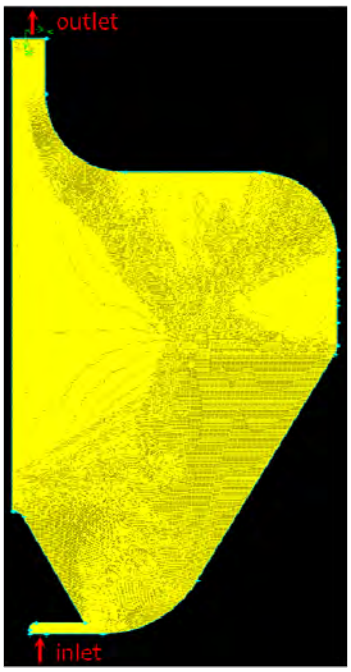


Fig. 140. HDCD axisymmetric geometrical meshed model (red arrows indicate inlet and outlet flow sections).

The culture medium (density = 1006.5 kg/m^3, viscosity = 1.003·10^{-3} kg/(m·s)) was simulated as Newtonian fluid, with inlet velocity values ranging in the interval 0.05 – 0.4 m/s, guarantying Reynolds numbers lower than 1000 at inlet section (laminar flow condition). At the wall, no slip-conditions were imposed.

For assessing the suitability of the geometry, the flow field (velocity and vorticity contours) was analyzed. Remarkable differences in the cell-seeded hydrogel distribution and suspension, and in the shear distributions arise due to variations in perfusion parameters. Results extracted from the computational analysis demonstrated the suitability of the geometrical design imposing a inlet velocity value of the culture medium of 0.3 m/s (Re = 900):

- Fig. 141 shows the color map of velocity magnitude values;
- Fig. 142 shows the color map of vorticity magnitude values.

The visual inspection of Fig. 141 and Fig. 142 allows to appreciate that buoyant stationary vortices establish within the culture chamber, that guarantee microgravity conditions and avoid sedimentation.

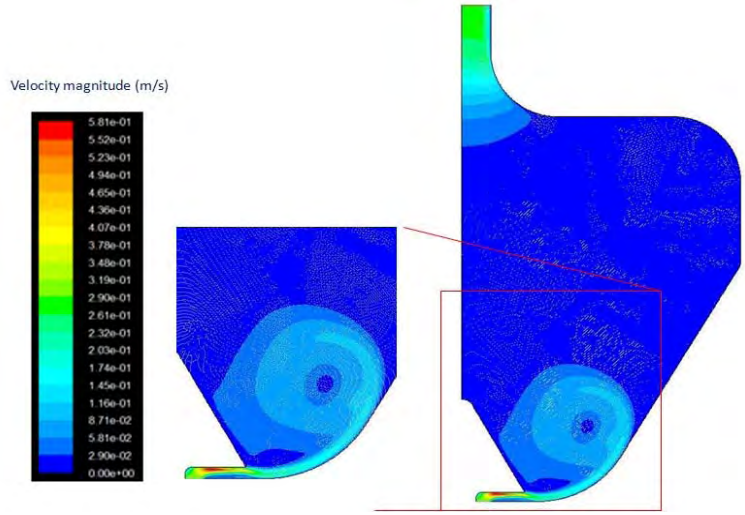


Fig. 141. Color map of velocity magnitude values (inlet velocity = 0.3 m/s).

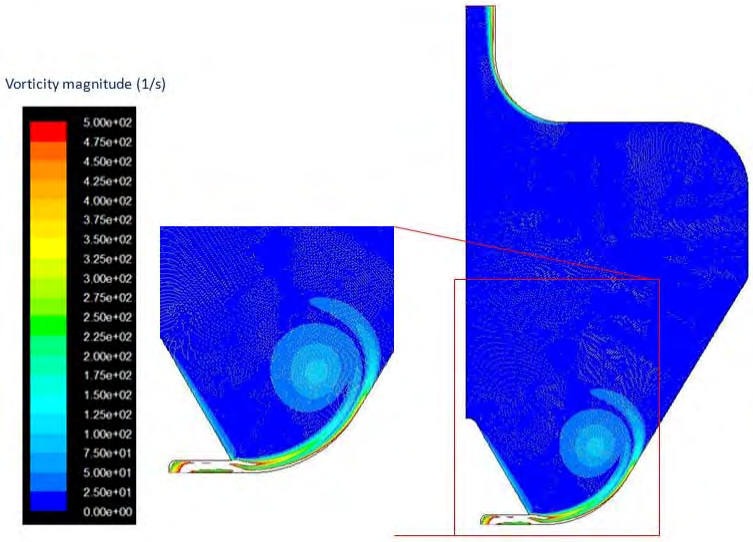


Fig. 142. Color map of vorticity magnitude values (inlet velocity = 0.3 m/s).

Moreover, in order to assess the presence of shear values critical for the cultured cells (shear critical value for cell damage = 2 Pa), the strain rate distribution was studied (Fig. 143). The maximum shear value, reached in the region of the culture chamber where cells are located, is 0.4 Pa, lower than the value for cell damage, and it was calculated by multiplying the maximum value of the strain rate, in the same region, for the viscosity of the culture medium.

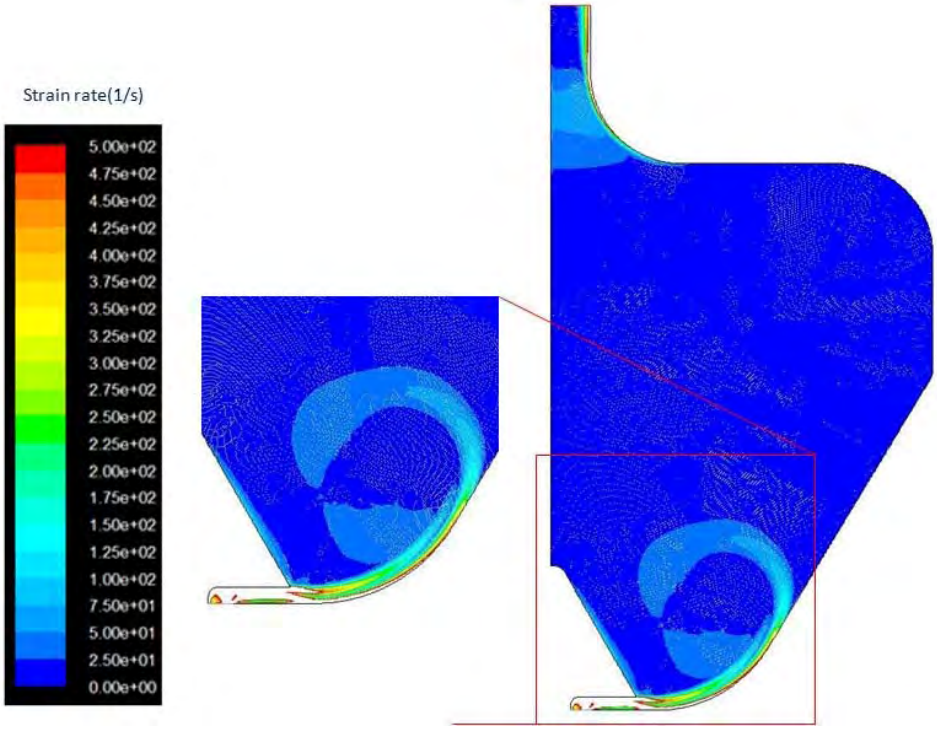


Fig. 143. Color map of strain rate values (inlet velocity = 0.3 m/s).

The results of the simulations demonstrate that the proper combination (1) of the fluid dynamic conditions establishing at the inlet vane of the chamber with (2) the shape of the side walls of the chamber itself give rise to flow separation, with the consequent formation of stationary buoyant vortices and of hydrodynamic forces the resultant of which balances the gravitational force, thus avoiding the sedimentation of cultured specimen at the bottom of the culture chamber. Moreover, the microgravity condition avoids shear values critical for the cultured cells.

The parameters used for the simulation will be used for setting the operating conditions for preliminary in vitro tests.

3. Context analysis and market

Given the preliminary studies described in Chapters 1 and 2, a strategy for developing scaffolds and bioreactors can be prepared. But, as in every field, even more in this field a deep knowledge of its context and its market is absolutely needed.

Tissue engineering products can be framed in the healthcare industry.

The healthcare industry is on the cusp of significant change: the rising cost of healthcare is placing a huge pressure on payer institutions and the recent financial crisis has placed an even greater focus on controlling this expenditure.

Although it may be worrying that healthcare costs are raising significantly, the result is that this is a sector that continues to grow even in challenging economic times: according to a CBO report[11], healthcare expenditure in US has tripled every twenty years since 1965.

Since both scaffolds and bioreactors to be developed are classified as medical devices, it is important to introduce a clear definition of the Medical Devices Field, which is a particular niche of the Health Market: it has a very large group of products which have the common objective to be devices for permanent support to patients suffering of long-term and/or chronic pathologies. This segment is more and more important in Europe, considering the impact on the healthcare expenditure and on health itself.

According to Eucomed data, the European Market of Medical Devices represent one-third of a global market of about € 187 billion, which makes the Europe the second largest market behind US, which represent nearly the 42%.

Even if this segment is not yet developed as in US, in Europe nearly 435.000 people are employed in the Medical Devices industry.

Its main characteristics are:

- a constant flow of innovation, achieved by large R&D investments;
- a short product lifecycle, with an average of 18 months, before an improved product becomes available;
- a very high concentration of the market share in the hands of few leaders and a high rate of SMEs[12] (nearly the 80% of the total).

These points, mainly the last two points, suggest that development of this kind of product by either a SME or a SME consortium can be feasible.

In the last few years the growth rate of this market has been higher than the pharmaceutical's one of nearly 6%, with rates of 10-20% in the cardiovascular and neurological segments.

Medical devices are usually divided in 12 cathegories and 10.000 generic groups, gathering a total of more than 500.000 products (Table 1).

[11] CBO, Congressional Budget Office, is the USA statistical office.
[12] SME is a Small Medium Enterprise; this definition is strictly assessed by EU directives.

Table 16: Categories of medical devices.

Code	Term	Examples
01	Active implantable technology	Cardiac pacemakers, neurostimulator, etc.
02	Anaesthetic and respiratory technology	Oxygen mask, anaesthesia breathing circuit, gas delivery unit, etc.
03	Dental technology	Dentistry tools, alloys, resins, dental floss, brush, etc.
04	Electromechanical med. tech.	X-ray machine, scanner, laser, etc.
05	Hospital hardware	Hospital bed, etc.
06	In-vitro diagnostic technology	Pregnancy, blood glucose, genetic tests, etc.
07	Nonactive implantable tech.	Hip, knee joint replacement, cardiac stent
08	Ophthalmic and optical tech.	Eye glasses, contact lenses, ophtalmoscope, etc.
09	Reusable instruments	Various surgical instruments
10	Single use technology	Syringes, needles, gloves, balloon catheters, etc.
11	Technical aids for disabled persons	Wheelchair, walking aid, hearing aid, electrical bed, etc.
12	Diagnostic and therapeutic radiation technology	Radiotherapy units

According to the Council Directive 90/385/EEC, and the Council Directive 93/42/EEC, "medical device means any instrument, apparatus, appliance, material or other article, whether used alone or in combination, including the software necessary for its proper application intended by the manufacturer to be used for human beings for the purpose of:

- diagnosis, prevention, monitoring, treatment or alleviation of disease;
- diagnosis, monitoring, treatment, alleviation of or compensation for an injury or handicap;
- investigation, replacement or modification of the anatomy or of a physiological process;
- control of conception,

and which does not achieve its principal intended action in or on the human body by pharmacological, immunological or metabolic means, but which may be assisted in its function by such means".

Moreover, devices may also be classified as[13,14]:

- active medical device: any medical device relying for its functioning on a source of electrical energy or any source of power other than that directly generated by the human body or gravity;
- active implantable medical device: any active medical device which is intended to be totally or partially introduced, surgically or medically, into the human body or by medical intervention into a natural orifice, and which is intended to remain after the procedure;
- custom-made device: any device specifically made in accordance with a duly qualified medical practitioner's written prescription which gives, under his responsibility, specific design characteristics and is intended for the sole use of a particular patient. Mass- produced devices

[13] Council directive 93/42/EEC of 14 June 1993 concerning medical devices and following modifications and integrations,

[14] Council directive 90/385/EEC on the approximation of the laws of the Member States relating to active implantable medical devices and following modifications and integrations.

which need to be adapted to meet the specific requirements of the medical practitioner or any other professional user shall not be considered to be custom- made devices;

- device intended for clinical investigation: any device intended for use by a duly qualified medical practitioner when conducting clinical investigation […] in an adequate human clinical environment.

The Medical devices segment differs from the Drug Market for various reasons, most importantly for regulatory requirements, time to market (usually faster for Medical devices than for drug), product life cycles (much faster for Medical devices) and competences/knowledge required for their development which is mainly chemical/pharmacological/biological for Drugs and a mix of chemistry/engineering/biology/physics/ for Medical Devices (Table 17).

Table 17: Comparison between medical device and drug market features.

Medical Devices	Drugs
Scientific knowledge	
Based on chemical, physical and systemic knowledge, and on genetic engineering	Based on chemistry and pharmacology
Use	
They can be used only where the personnel has the necessary knowledge, and the medical structure is adequate	They can be used in almost every environment, independently of the hospital structure
R&D	
R&D activities are focused mainly on product engineering	R&D activities are focused on the drug, and are based on the evaluation of polymers and molecules

Medical Devices	Drugs
Product Life Cycle	
Permanent innovation, short product life	Fidelity on the product, long product life
Regulation	
General safety on the production process must be demonstrated	Registration and single-product control procedures are necessary
Market concentration	
Few big enterprises and a great number of SMEs	Multinational enterprises

In 2007 there were almost 100.000 people on transplant waiting-list in US and many would die while waiting for available organs, others would be refused due to contraindications and others would suffer from chronic rejection while all of them would depend on immunosuppressive treatment.

In nature we have examples of organisms capable of regenerating significant portions of damaged tissues: the amphibian newt (limbs) and the zebra fish (heart) among others. The cellular mechanism consists of the

re- entry of differentiated cells located next to the site of injury into the cell cycle, followed by their differentiation and proliferation to form new tissue.

Several mechanisms may account for the inability, in humans, to do the same: aging, pathologic state, inflammatory response, limited number of resident stem cells and so on.

So, in the human case, tissue engineering may be defined as the method to promote the body healing itself thanks to the delivery, into the appropriate sites, of cells and biomolecules with supporting structures.

A broad spectrum of diseases is already addressed with tissue engineered products either in clinical use or in an experimental phase, and they include breast reconstruction, bone/cartilage/tendon and vertebral disc substitutes, corneal replacement, cosmetics and cardiovascular applications.

The Tissue Engineering industry started in 1985 with the work of four independent laboratories at the Massachusset Institute of Technolgy (MIT) at Cambridge, US. The industry continued to expand throughout the 1990s and reached its peak in 2001: the sector consisted of more than 70 companies that employed nearly 3300 people and had a combined annual expenditure of more than $ 600 million. In his 2001 report, Michael Lysaght estimated that, between 1990 and 2000, $ 3.5 billion was invested worldwide in tissue engineering and that 90% of that was private sector finance: such imbalance in funding had a profound impact on the direction of the research and on the timeframe under which results had to be delivered (238).

By this stage, the industry was starting to run into problems. The most serious of these was underperformance: the first products, although mostly approved by the regulators, just did not behave as originally intended, once they entered the clinic.

In the absence of commercially successful products or profitable out licensing deals, tissue engineering companies struggled to make money and their overdependence on private investment became a real problem.

In autumn 2002, the two pioneering corporations in this sector, Organogenesis and ATS, each valued at their peak at approximately $ 1 billion, both went bankruptcy within a month of each other. Ortec downsized considerably and Genzyme Tissue Repair merged with another Genzyme division to form Genzyme Biosurgery. Between 2000 and 2002, activity in the sector fell by 50% and 800 full-time employees were lost. Furthermore, the capital value of publicly traded tissue engineering corporations dropped by almost 90%, from $ 2.5 billion at the end of 2000 to $ 300 million by the end of 2002.

Today, business plans have become more realistic: modest but achievable profits based upon reasonable investment have become more attractive than long-shot blockbusters. These trends toward a risk-management-based business model have been accelerated by the entry of very large firms into the field (Boston Scientific, Baxter, Medtronic, Genzyme, Johnson & Johnson).

The mass public and political support has created a demand for products that is unparalleled in the history of regenerative medicine. Today the approach is that the research and the scientific discoveries are made by academia founded by public money. This frees the companies to invest in translation of science into commercial product.

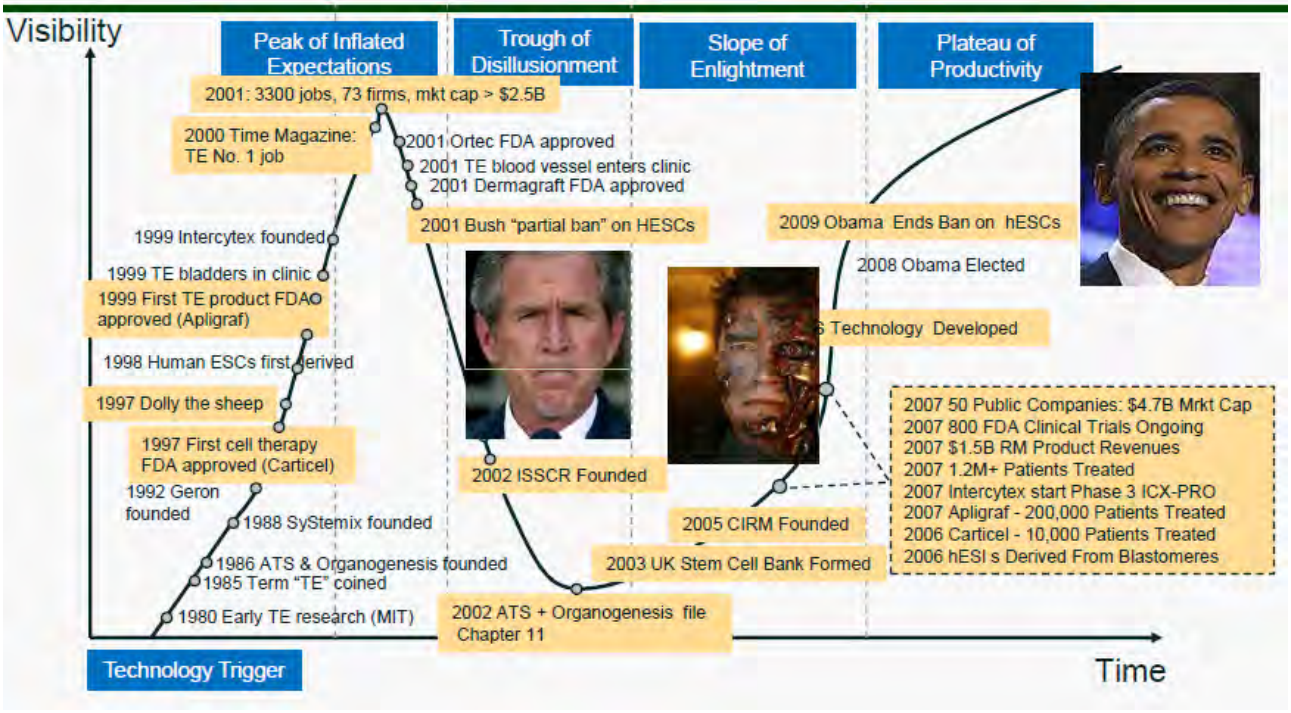


Fig. 144: Gartner curve, associated with events (239).

Current targets include heart failure, spinal-cord injury, stroke and diabetes. Hundreds of patients are being enrolled in various cardiac studies all round the world to test the merits of this approach.

To date, more than 1.2 million patients have been treated with regenerative medicine products.

The leaders of the new Regenerative Medicines are companies significantly different from the pioneers. No longer are they made up of great visionary scientists, but are instead seasoned businessmen focused on building great companies.

A good example is Organogenesis, which is headed up by Geoff MacKay (ex Novartis). In 2006, an important milestone for the industry was reached, under its new management; Organogenesis became the world's first profitable regenerative medicine company.

So, it is possible to say that the Tissue Engineering market is maturing:

- it is rapidly expanding ($ 3.6 billion in 2008 and $118 B estimated in 2013);
- it is facing a dramatic revenue growth ($1.5 billion in 2008);
- the worldwide funding for research is increasing (in 2009 was $1.5 billion, and it is expected to arrive at $ 14B in 10 years);
- there are over 900 clinical trials;
- 26 products on the market and 94 in development;
- more than 40 public companies active on the market (240).

The market is, in fact, at a crucial inflection point: the regenerative medicine is maturing after 40 years of development, and research programs are proliferating with an increased governmental funding. This is also due to a market pull, because the population is ageing and by 2035 one person over two will be over 65

years old and there are large unmet medical needs. Surely, a great boost has also been given by the pharmaceutical companies, which saw the potential of such market and actively engaged in it.

In 2008, tissue engineering and cell based therapies were mainly present in the areas of skin, cartilage and bone: the total market, covering the areas of skin, bone, cartilage, dental and organ regeneration or replacement, was valued about 11.5 billion dollars, with a compound annual growth rate of 25.7% (239).

Moreover, the maturity profile is low, because the pipelines are based mainly on early clinical trials (phase I and II). Very few pharmaceutical laboratories or medical device companies are active in this market at the moment.

Acellular products, like bioreactive bone grafts and biomaterials (so, scaffolds), predominate at the moment because they avoid the technical challenges and regulatory issues implicit in selling a product containing living cells.

Firms today have lessened their dependency on venture capitals (VCs) by seeking funds from other sources, including angels, Debt, or other forms of public–private cooperation. Outsourcing, even of manufacturing, is far more common.

Firms that can operate in minimally regulated areas or with more conventional products have a significant advantage in terms of ‘‘time to market.’’ This explains the commercial prominence of cord blood banking and of acellular biomaterials.

In the early to late 1990s, commercial tissue engineering was almost exclusively conducted in the United States. The trend toward internationalization began around 2000, and has continued ever since. Today, commercial activity in the field is about 55% U.S.-based and the remainder outside the United States. International diversification is clearly a benefit, as it provides the field with broader access to talent, markets, business models, and governments.

At present time, more than 25 products are on the market and about 100 in development (241); the therapeutic area includes bone, skin, soft Tissue (adipose) and cartilage and the big companies involved are Medtronic, LifeCell, Genzyme, Organogenesis, Osiris, Cytori[15]. Table 18 shows the top ten products in commerce.

Table 18: Top ten tissue engineering companies and their products.

Company	Product	Product Type	Therapeutic Area	Indication	Launch	2007 WW Revenue	’06-’07 Rev Growth
Medtronic	**Infuse**	**Growth factor w/matrix**	**Bone**	**Spinal fractures, orofacial fractures, open tibial fractures**	**2002**	**~$700ME**	**18%**
LifeCell	**Alloderm**	**Allogeneic acellular matrix**	**Skin**	**Skin replacement/ hernia repair**	**1994**	**$167.1M**	**40%**
Genzyme	**Carticel**	**Autologous cell based**	**Cartilage**	**Knee repair**	**1995**	**~$88ME**	**~30%**
Stryker	**OP-1**	**Growth factor**	**Bone**	**Humanitarian Device**	**2005**	**~$80ME**	**60%**

[15] Company 10K information, Frankel Group Analysis.

		w/matrix		Exemption for spine fusion & long bone fractures			
RTI	Spinal Implants	Allogeneic Acellular matrix	Bone	Spinal fractures	1991	$41.1M	17%
Organogenesis	Apligraf	Allogeneic Neonatal cells w/matrix	Skin	Diabetic skin ulcers	1998	~$30ME	10%+
Advanced Biohealing	Dermagraft	Allogeneic Neonatal cells w/matrix	Skin	Diabetic skin ulcers	1997	~$20ME	10%+
Integra Lifesciences	Various	Allogeneic acellular matrix	Skin	Skin repair / replacement	2001	~$20ME each	25%
Osiris/ Nuvasive	Osteocell	Allogeneic cell based	Bone	Fracture repair	2005	$15.2M	83%
Cytori	Celution	Autologous cell based	Soft Tissue (adipose)	Reconstructive Breast Surgery	2008 (ex-US)	~$10-12M*	N/A

Fig. 145 and Fig. 146 show the situation in 2009 of tissue engineering product development stages and their distribution over therapeutic area respectively.

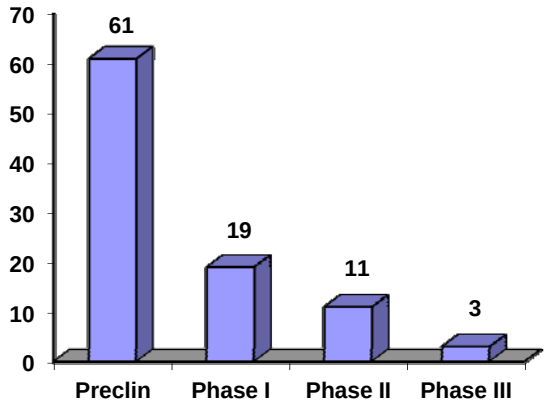


Fig. 145: Products in development by phase (94 products) (240).

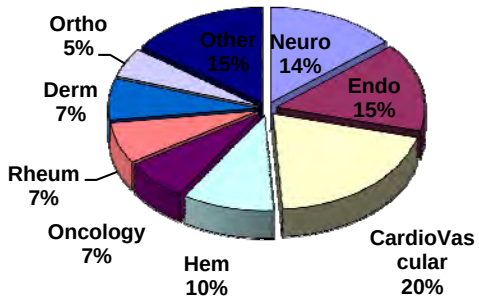


Fig. 146: Products in development by therapeutic area (240).

Trends that include aging, baby boomers, rising expectations for medical cures, and increasing health care costs for existing healthcare treatments have created a large potential market for breakthrough medical solutions led by Regenerative Medicine (RM).

Another important driver is the increase of the healthcare cost (Fig. 147).

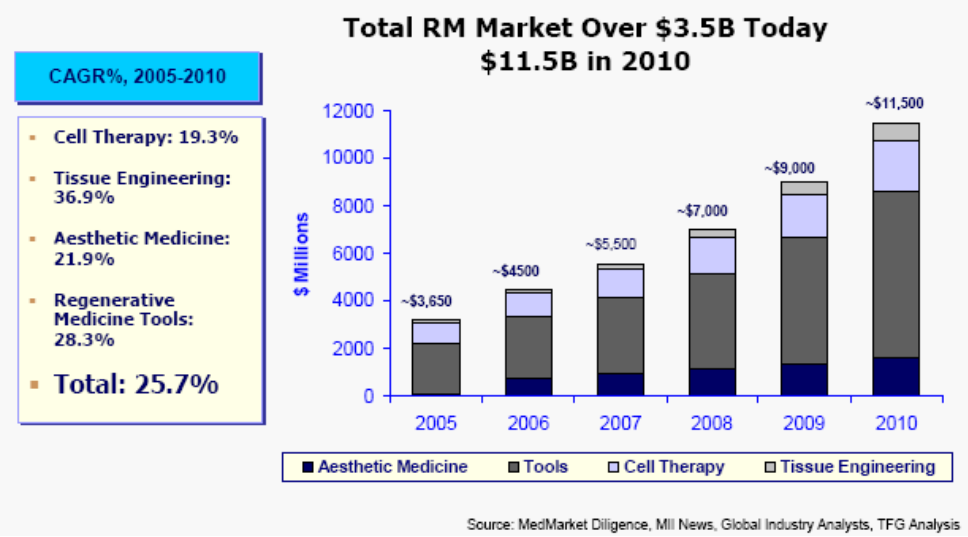


Fig. 147: Regenerative medicine (RM) market projection (2005- 2010).

Regenerative medicine could cause two types of economic impacts:

- health–related benefits (cost savings in healthcare, increases in productivity, and the reduction of premature deaths);
- attributable economics benefits (focused on local and regional economic development, including the economic impact of public expenditures).

The potential savings in the sole U.S. are estimated to be $ 250 billion per year. In a broader context, it may be useful to know that Japanese government has invested $ 831 billion in the Kobe Medical Industry Development Project to advance medical care and a key component is the regenerative medicine fields; the China's Ministry of Science and Technology will spend between

$ 63 and $ 250 million on regenerative medicine over the next few years while South Korea and Singapore are allocating funds to boost stem cells research in the next few years .

Medtech Insight analyzed the market opportunity in the U.S., evaluating the potential patients caseloads: it is reported a potential market of $ 85.4 billion in the sole U.S., of which $ 13.3 billion only for the cardiac and vascular products; this evaluation is based on a potential patient caseload of 6.5 million (Fig. 148).

Market Segment	Potential Market, 2006	Potential Market, 2016	CAGR (2006-2016)
Cardiac and Vascular Products	$13,321M	$17,447M	2.7%
Musculoskeletal Products	29,160	36,386	2.2
Neurologic Products	9,278	10,977	1.7
Urologic Products	7,158	8,612	1.9
Organ Regeneration Products	10,910	16,443	4.2
Skin Replacements and Substitutes	15,642	19,393	2.2
Total	**$85,469M**	**$109,258M**	**2.5%**

Source: Medtech Insight

Fig. 148: Potential market by segment.

3.1. A spot on cardiovascular market

Number one cause of death or permanent disability in developed countries is represented by Cardiovascular disease.

Estimated 81.100.000 Americans adults (more than 1 in 3) have one or more cardiovascular disease: of these, 38.100.000 is estimated to be age 60 or older.

On the total, nearly 8.500.000 people suffer of myocardial infarction and 5.800.000 of heart failure: thus, it is possible to say that a death every three is due to cardiovascular disease, and in every year since 1900, cardiovascular disease accounted for more deaths than any other major cause of death in the United States: since for Europe the last data are of 2008, it is possible to suppose that the trend for the EU is similar to that of US.

The estimated direct and indirect 2010 cost of cardiovascular disease is $ 503.2 billion, while for heart failure is $ 39.2 billion, for the sole US area.

From 1996 to 2006, the total number of US cardiovascular operations and procedures increased 33% from 5.444.000 to 7.235.000 annually (AHA computation, Fig. 149).

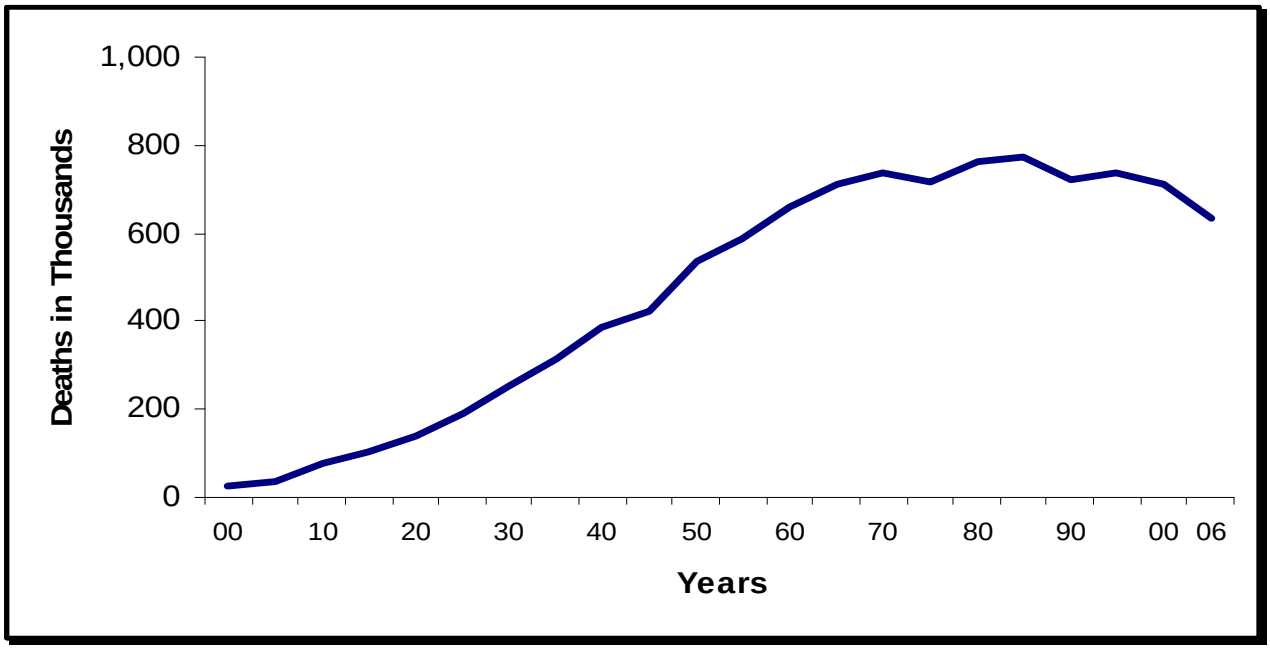


Fig. 149: Deaths from cardiovascular diseases (US, 1900-2006; source: NCHS and NHLBI).

The main cause of death within CVD is Coronary Heart Disease (CHD or ischemic disease), which represent 42 % (7.2 million) of all CVD deaths in the world .

Althought CVD death rates are declining in the developed countries, they are increasing in every other region of the world and by 2020 heart disease and stroke will become the leading cause of both death and disability worldwide, with a predicted number of deaths of nearly 20 million per year.

In the enlarged EU, in 2007, an average of 155.050.000 million people suffered of CVD and the financial burden for EU health care systems related to this group of diseases has been estimated to be just under € 110 billion (2006). This represents a cost per capita of € 223 per annum, around 10% of the total health care expenditure across the EU.

The cardiovascular medical device segment is the richest out of the many medical devices segments: overall sales in the global market devices were nearly $76.7 billion in 2008, which is projected to increase substantially to $84.6 billion in 2010. By 2015, it will increase to $97.3 billion, at 5 year compound annual growth rate (CAGR) of 2.8%. The largest segment interventional cardiovascular devices market was nearly $60 billion in 2008; which will reach $66 billion in 2010: that is expected to rise to $74 billion in 2015, a CAGR of 2.4%[16] (Fig. 150).

[16] BCC research (BCC Research is a publisher of technology market research reports and reviews, and of technical newsletters, including reports on automotive sensors, biopesticides, geosynthetics, DNA sequencing, carbon capture and storage technology, the market for probiotics, and global medical markets for nanoscale materials and devices).

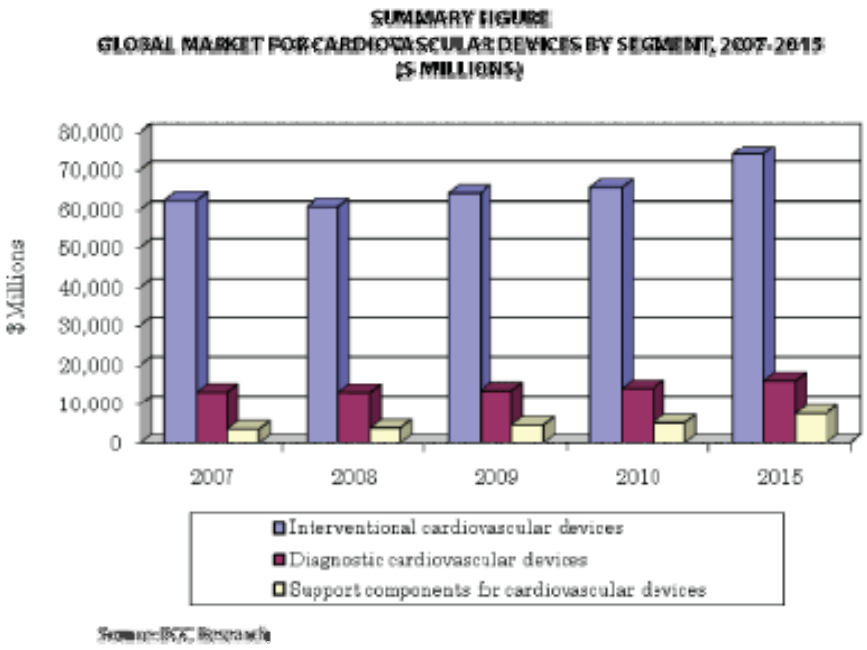


Fig. 150: Global data for cardiovascular segment.

CardioVascular Diseases (CVD) encompass a number of specific illnesses such as coronary heart disease, heart failure, arrythmias, stroke, arterial and pulmonary hypertension, cardiomyopathies, and these are one of Europe's largest health problems.

Despite dramatic medical advances over the last few decades, cardiovascular diseases, as above reported, remain the leading cause of death in the European Union. They account for 30% of deaths worldwide, according to the World Health Organization[17] and 42% in the EU (according to the British Heart Foundation[18]).

Tissue Engineering may be seen as a possible solution for patients which are currently treated with palliative approaches (drugs) or with medical device therapies who remove the cause of the disease (i.e. coronary obstructions), but not the consequences of the disease (myocardial degeneration /ischemia due to lack of perfusion) who often lead to heart decompensation and failure. Therapies that would cure the consequences of coronary disease, for example, would reduce patients re-hospitalization costs, costs of disability treatments and, for transplanted patients, the risk of rejection.

Tissue engineered medical products are expected to play a major role in the future of cardiovascular bypass surgery, heart valve replacement, venous valve repair, and other intravascular surgeries. Within the field, there are a wide range of applications for myocardial and vascular regeneration, including cell-seeded scaffolds for heart valve and myocardial regeneration or the creation of arterial grafts, cryopreservation, decellularizing allogenic tissues such as matrices, the delivery of genes and proteins to stimulate the growth of new blood vessels (angiogenesis), and gene therapy to prevent the re-narrowing of treated vessels (restenosis).

At the moment, about 20% of the total regenerative products in development are related to the cardiovascular business.

[17] www.who.org
[18] www.heartstats.org

The key driver for the tissue engineered cardiac and vascular products market is the need for improved methods to treat CVD, in particular methods to treat the damaged myocardial tissues.

Nowadays there are more different companies involved in developing tissue engineering products in the cardiovascular segment, and the majority of them are focused on products for the coronary artery disease with cell based technology. We will make a specific review of these companies in the session related to Competition (Chapter II.4)

The most of companies are working at the development of new cells and gene therapies, while fewer are concentrated on scaffold (even if this area seems to be the most promising and more and more R&D groups and industries are incrasing efforts in this sense).

Despite the great effort, being the myocardium a much more complex tissue than bones and or skin, no product are currently available in the market with the specific claim of tissue regeneration.

3.2. Regulatory

Generally, medical products can be classified in 3 categories:

- Medical Devices
- Drugs
- Biologics

The so called “Advanced Therapy Medicinal Products” fall in the Drugs category in terms of Regulatory, even if it is an area which is submitted to continuous evaluations and frequent updates/changes of the regulatory approach carried on by both EMA and FDA.

Definitions:

According to the Directive 93/42/EEC, Medical Devices are “any instrument, apparatus, appliance, software, material or other article, whether used alone or in combination, including the software intended by its manufacturer to be used specifically for diagnostic and/or therapeutic purposes and necessary for its proper application, intended by the manufacturer to be used for human beings for the purpose of:

- diagnosis, prevention, monitoring, treatment or alleviation of disease;
- diagnosis, monitoring, treatment, alleviation of or compensation for an injury or handicap;
- investigation, replacement or modification of the anatomy or of a physiological process;
- control of conception

and which does not achieve its principal intended action in or on the human body by pharmacological, immunological or metabolic means, but which may be assisted in its functions by such means”.

So, typically, the medical device function is achieved by physical means. It may be assisted in its function by pharmacological, immunological or metabolic means, but as soon as these means are not ancillary with respect to the principal intended action of a product, the product no longer fulfils the definition of medical device .

In the European Union Drugs and Advanced Therapy follow the same classification and legislation, carried out by the Directive 2001/83/EC and following amendments. In particular, Directive 2009/120/EC, amending the above mentioned Directive, regulates the advanced therapy medicinal products.

- Medicinal product (def. Dir. 2001/83/EC):
  1. Any substance or combination of substances presented as having properties for treating or preventing disease in human beings or
  2. Any substance or combination of substances which may be used in or administered to human beings either with a view to restoring, correcting or modifying physiological functions by exerting a pharmacological, immunological or metabolic action, or to making a medical diagnosis.

In order to obtain an authorization to place a medicinal product on the market an application shall be made to the competent authority of the Member State concerned.

The application shall be accompanied by the results of:

- pharmaceutical (physico-chemical, biological or microbiological) tests;
- pre-clinical (toxicological and pharmacological) tests;
- clinical trials;

a detailed description of the pharmacovigilance and, where appropriate, of the risk management system which the applicant will introduce .

Moreover, the Regulation (EC) 1394/2007 (amending Dir. 2001/83/EC) defines all the kinds of medicinal products which may fall under the broader definition of Advanced Therapy Medicinal Products:

- Advanced therapy medicinal product means any of the following medicinal products for human use: a) a gene therapy medicinal product; b) a somatic cell therapy medicinal product; c) a tissue engineered product
  - Tissue engineered product means a product that: contains or consists of engineered cells or tissues AND is presented as having properties for, or is used in or administered to human beings with a view to regenerating, repairing or replacing a human tissue. It may contain cells or tissues of human or animal origin, or both. It may also contain additional substances, such as cellular products, bio-molecules, bio-materials, chemical substances, scaffolds or matrices.
    - Cells or tissues shall be considered engineered if they fulfil at least one of the following conditions: a) they have been subjected to substantial manipulation, so that biological characteristics, physiological functions or structural properties relevant for the intended regeneration, repair or replacement are achieved; b) the cells or tissues are not intended to be used for the same essential function or functions in the recipient as in the donor.
  - Combined advanced therapy medicinal product means an advanced therapy medicinal product that fulfils the following conditions: 1) it must incorporate, as an integral part of the product, one or more medical devices or one or more active implantable medical devices and 2) its cellular or tissue part must contain viable cells or tissues or 3) its cellular or tissue part containing non- viable cells or

tissues must be liable to act upon the human body with action that can be considered as primary to that of the devices referred to[19].

We can observe that Advanced Therapy medicinal products have stricter requirements than Drugs and Medical Devices.

In fact, the marketing authorization holder of an Advanced Therapy medicinal product, shall establish a traceability system for the product and for its starting and raw materials, including the substances that may come in contact with the cells or tissue that it contains: the product must be traced from the sourcing to the final user.

If an advanced therapy medicinal product contains a device, the requirements become more tightening, because there will apply not only the new requirements on the advanced therapy products, but also the ones referring to the medical devices[20].

So, during the development pathway of tissue engineering products, it is fundamental to keep in mind these rules and not to cross the frontier of Advanced Therapy medicinal products; if not, long time and large investment would be needed, making any SME fail.

Medical devices are classified by directive 93/42/EEC in various categories which requires progressively more stringent pre-clinical and/or clinical investigation in order to get approved:

- Class I (a,b)
- Class II (a,b)
- Class III

It is now important to classify tissue engineering scaffolds and tissue engineering bioreactors in order to understand how difficult their development is from a regulatory point of view.

Let's point out some of the rules of annex IX of the aforementioned Medical Device Directive 93/42/EEC.

Non invasive medical devices:

Rule 1: all non-invasive devices are classified as class I, unless one of the following rules applies.

Rule 2: all non-invasive devices intended to canalize or store blood, body fluids and tissues, liquids or gases intended for transfusion, subministration or introduction in the body, are classified as class IIa if:

- they can be connected to active medical devices belonging to class IIa or higher;
- they are intended to be used for storage or canalization of blood or other body fluids or storage of organs, parts of organs, body tissues.

In any other case, they are classified as class I.

.....(*omissis*).....

Invasive medical devices:

[19] Regulation (EC) 1394/2007 (amending Dir. 2001/83/EC).
[20] As discussed during BIOSCENT 1st Exploitation Meeting, Madrid, 10th of February 2010.

.....(*omissis*).....

Rule 8: all implantable devices and long-term surgically invasive devices are in Class IIb unless they are intended:

- to be placed in the teeth, in which case they are in Class IIa,
- to be used in direct contact with the heart, the central circulatory system or the central nervous system, in which case they are in Class III,
- to have a biological effect or to be wholly or mainly absorbed, in which case they are in Class III,
- or to undergo chemical change in the body, except if the devices are placed in the teeth, or to administer medicines, in which case they are in Class III.

.....(*omissis*).....

According these rules, bioreactors, that are non-invasive medical devices, can be classified as class I when used as test machines in a laboratory and as class IIa when used to grow body tissues to be implanted. Both classes have an easy certification just aimed at achieving a standard CE mark by a notified body.

On the other hand, scaffolds are IIb when non resorbable, unless they are placed in teeth (in which case, they are class IIa); when resorbable, they are class III, and this is the worst case a SME can affordably face.

While bioreactor development give no regulatory problem at all, it is absolutely not advisable to propose the development of cell seeded scaffolds, because this would make this product fall in the already mentioned Advanced Therapies medicinal product category. So, a SME should focus its effort in developing non seeded scaffolds, better if in the dental field, whose regulation is less tight.

Some additional certification routes can be pointed out.

For example, medical devices with no medicinal substances with ancillary action will require a certification by a medical device dedicated notified body following directive 93/42/EEC and following modifications and integrations. If the medical device contains a medicinal substance with ancillary action, it will continue to be classified as medical device, but together with the certification by a notified body, following directive 90/385/EEC, it usually requires additional preclinical tests (e.g.: toxicity; release-kinetics, corrosion etc…) evaluated through a consultation process with a competent authority.

If the medical device contains proteins, its classification (as medical device or pharmaceutical product) depends on the mode of action of the proteins:

- if their action is purely physical: e.g. binding of cells by physical means to extracellular matrix proteins as e.g. by RGD-sequences: it still is a medical device of class III.
- if the action is by pharmacological, immunological or metabolic means the protein itself will be treated as a medicinal substance;
- if this action of the protein is ancillary, the product may be a medical device;
- if it is the main action it is not an advanced therapy product but a pharmaceutical product, a so-called “Biological”.

So, if the use of proteins (ligands, growth factors...) is needed in order to promote cell activity once the scaffold is implanted, this product will be classified as medical device class III as well, and no regulatory problems will occur.

If medical devices contain non-viable materials of animal origin, directive 2003/32/EG applies. Practically this means, that the Notified Body needs an accreditation for this directive. A Summary Evaluation Report on the Assessment of a Medical Device by the Notified Body for Conformity with Council Directive 93/42/EEC and Commission Directive 2003/32/EC needs to be sent to the national competent authority by the notified body and there are some special requirements for risk analysis.

The sterilization process has no impact on the classification of the products as medical device or advanced therapeutic product, but, as some of the devices containing proteins may not be sterilized by the methods as e.g. by heat, or radiation or ethylene oxide, the requirements for aseptic processing should be considered at least for some production steps. There is a complete set of official standards which regulate the sterilization matters. This should considered in the evaluation of development and production costs.

3.3. Bioreactor exploitation pathway: the challenges

As already stated, in the last couple of decades, the appealing possibility of combining living cells with suitable carriers for the regeneration of damaged or lost tissues and organs has promoted interaction among scientists, engineers, clinicians and business people, leading to the establishment and progressive consolidation of the field of tissue engineering.

Despite significant achievements and enormous clinical demand, the clear need for viable engineered tissue products that are broadly available to patients as part of the routine toolkit of medical treatments still remains unsatisfied. On the one hand, this might be because of relatively limited establishment of prospective, randomized clinical studies demonstrating reproducibly superior effectiveness of engineered grafts compared to conventional treatments. On the other hand, it has been proposed that addressing manufacturing-related issues is the key for success of cell-based engineered products (241). Indeed, as in other biotechnology applications (e.g. the production of antibodies or molecular vaccines), successful clinical use of engineered tissue products, as well as their commercial exploitation, might be critically dependent on the introduction of bioreactor-based manufacturing systems (3).

Bioreactors, intended as a means to generate and maintain a controlled physicochemical culture environment, indeed represent a key element in the automated, standardized, traceable, cost-effective, safe and regulatory- compliant manufacture of cell-based products or engineered grafts for clinical applications (242), (243).

So, it is important to address the scientific, regulatory and commercial challenges that are hampering the bioreactor-based translation of tissue engineering strategies into clinical products and, based on these issues, propose a roadmap for the development of a more advanced paradigm. This paradigm is fundamentally rooted in the perspective that sensor-based bioreactors must be deployed and validated throughout the product development pipeline, from the initial concept until manufacture of the implant.

3.3.1. Scientific challenges

The introduction of bioreactors in the field of tissue engineering was initially considered as a means to apply defined patterns of physical forces, aiming at regulating and improving the mechanical functionality of the resulting engineered tissues (244). This approach favoured scientific understanding of

mechanoregulation processes in developing tissues and emphasized the importance of controlled physical conditioning in tissue engineering.

However, the need to apply mechanical forces *in vitro* to generate functional grafts is still controversial. In a few cases, graft functionality might need to be developed prior to implantation, such as for engineered blood vessels (245) and heart valves (246). But it is becoming increasingly clear that tissue maturation could be more efficiently induced by the physiological, biochemical and mechanical cues of the body as an "*in vivo* bioreactor" (247), provided that starting material, implanted *in situ*, has an appropriate quality.

The use of a mechanical loading regime that correctly emulates the dynamic physiology of the body to generate a fully functional tissue will probably make the manufacturing process too complicated too expensive, so, at this level of technology, impractical. In principle, compared to functionalized materials and drug delivery medical devices, cell-based grafts have the potential to provide superior clinical outcomes because of the multivalent biological activity of cells (multiple growth factor release, self-contribution to tissue regeneration) and/or of the extracellular matrix deposited (efficient storage and release of morphogens, physiologically functional mechanical properties).

But, engineered products will only be a viable and competitive alternative to upcoming off-the-shelf innovations in regenerative medicine (241) if they are manufactured with reproducible properties, a fundamental prerequisite for consistent clinical outcomes and validation.

This important target is mainly hampered by the intrinsic variability, often in the behaviour of human cells from different donors (248), and by the sensitivity of cells to the culture environment.

Whereas bioreactor-based tissue engineering has typically been focused on improving the functionality or maturation stage of the resulting engineered grafts, the potential for controlled sensor-based bioreactor systems to minimize process and product variability must be considered. If the culture parameters are monitored and controlled, bioreactors can help to standardize both the processes required and the resulting engineered tissue and ensure that automated protocols are compatible with regulatory and commercialization requirements (Table 19).

On the other hand, because of streamlining of tissue culture and bypassing of operator-dependent procedures, bioreactor-based systems have the potential to increase the stability of the graft manufacturing process (249), (250).

Table 19: Opportunities and challenges for developing bioreactors.

Scientific aspects **Opportunity**: Sensor-based bioreactors provide possibility to regulate bioprocesses in order to minimize process and product variability. **Challenge**: Further scientific insight is required to maximize the utility of bioreactors in providing engineered tissues with duplicable properties.
Regulatory aspects **Opportunity**: Bioreactor-based processes (e.g. monitoring and data management systems) can offer a high level of compliance to safety guidelines. **Challenge**: Equivocal regulatory guidelines currently interfere with a careful design of bioreactors that comply with clear specifications.
Commercial aspects

Opportunity: Automated bioreactor systems can deliver safe and standardized production of engineered tissues.
Challenge: The way of commercializing engineered tissue products are not well assessed, and this results in uncertainties related to markets, regulatory approval, and clinical adoption.

3.3.2. Regulatory challenges

The clinical introduction of engineered tissue products is likely to involve significant regulatory oversight with an additional degree of complexity compared to policies and guidance recently implemented at different international levels for cell therapy (251), (252), (253).

Whereas manufacturing strategies based on conventional manual cell-culture techniques might face difficulties in complying with the new regulatory framework, bioreactor-based manufacturing processes inherently involve automation and reproducibility that facilitate compliance with regulatory objectives. In particular, monitoring of process parameters and specific properties of the developing/final graft provide a higher level of traceability of key manufacturing data related to identity, purity and potency of the implant.

Moreover, by minimizing the manual procedures required for operator handling of cells and tissue constructs, automated bioreactor systems with control platforms and software-based data management will facilitate compliance to safety regulations.

Ultimately, a stand-alone, fully automated and closed system would provide a good manufacturing practice (GMP)-compatible manufacturing unit complete with environment control, full operational traceability and failsafe protection measures that go well beyond simplistic automated cell culture. In essence, this is a type of GMP-ina-box concept with the potential to reduce dependence on large, costly and sometimes not easily available regulatory- compatible facilities (Table 19).

To translate these opportunities into effective ways to facilitate more widespread use of engineered tissue products in the clinic, we foresee commonly underestimated difficulties and challenges related to the practical details of implementation. First, the often rather undefined mode of action of engineered tissues and the limited availability of potency markers at a tissue level mean that pragmatic testing protocols that can be realistically deployed in the production process are difficult (see scientific aspects).

Issues related to the lack of certainty that the product output provides the expected patient benefit could hamper credible plans for validation, which guarantees performance with a high degree of confidence in the absence of direct data. Validation of production processes is pivotal to regulatory compliance and needs considerable attention in preparation for regulatory review.

Clinical deployment of bioreactors for the preparation of engineered tissues inherently depends on safe, reliable and user-friendly operation of the system in production environments. Fundamental to any regulatory assessment is a comprehensive analysis of the product, process, and environment risk, along with specific implementation of risk mitigation practices to address critical risks. Risk assessment is based on occurrence, severity and detection, with all factors playing a crucial role in identifying key nodes for active risk management.

As for other fields of biotechnology, the use of standardized and automated systems (in this case bioreactor-based manufacturing systems) means that critical risk factors associated with operator handling and the production process can be successfully mitigated.

However, an overview of risks and risk control for production of engineered tissues in bioreactor systems (Table 20) indicates the complexity and wide range of processes involved. Indeed, regulatory authority scepticism on the implementation of bioreactor-based graft manufacturing could be tempered by introducing a sound risk assessment early enough in product development.

Beyond the characterization and mitigation of risks associated with automated tissue engineering, regulatory approval of bioreactor systems for the production of cell based implants for clinical use will be highly dependent on the compliance of the surrounding production environment.

For facilities with pre-approval for cell manipulations, integration of additional equipment to further culture the implant would be significantly more straightforward (although appropriately challenging on the details of the process) than implementation in the more open environment of a specialized clinic. Uncertainties in the interpretation of a closed system, especially considering the sampling necessary for off-line monitoring (e.g. for sterility tests) and the need for direct intervention based on out-of-specification signals, make it difficult to identify precise details of the practicality of the GMP-in-a-box concept from a regulatory or safety standpoint.

Table 20: Risks and mitigation for bioreactor-based manufacturing systems

Risk	Risk control/mitigation
Biology	
Contamination and infection	• Closed sterilized system • Inputs sterile/clean when introduced • Contamination testing on every lot (individual patient level)
Toxicity/bioburden/pyrogens	• Bioreactor and related fluid contact surfaces selected from USP grade • Pre-test bioreactor system with target cell type • Validate production bioburden and pyrogen levels
Shear stress-induced cell damage	• Bioreactor system designed with benefit of fluid modelling • Validate operational sequence and maximum flow rates with target cell type
Compromised cell vitality and performance	• Biosensors for strict maintenance of culture conditions • Media and reagent sources refrigerated while on-line • Adaptive software to minimize effects of donor variability
Insufficient final cell population for clinical objective	• Establish gateway specifications in multi-step bioprocesses • Automate monitoring of cell behaviour to trigger cell collection
Sterilization failure	• Validate according to established international standards
Inadequate packaging shelf life	• Validate according to established international standards
Bioreactor/instrumentation	
Failure of disposable cassette in ensuring consistent and error-free operation	• Direct attachment of cell/tissue input container to minimize manual handling • Multi-layers to input container to reduce contamination risks • High level of process bioreactors visibility for operator verification of steps • Biological pathway entirely closed within cassette, zero transfer to instrument • Sampling ports for sterile withdrawal of samples (e.g. microbial testing) • Output container designed for direct transfer to clinical setting
Sensor reliability issues	• Sensor selection enables accurate monitoring for ex-vivo period without fouling • Operational back-up (dual and alternate monitoring)

	• Validate sensor tolerance to sterilization protocol • Validate sensor shelf life when incorporated into • cassette
Electromagnetic interference (EMI) and Electrical safety issues	• Test EMI emissions and sensitivity • Meet legislative standards for safety
Software	
Code error	• Design code according to standards • Rigorous code validation procedures
Software corruption	• Standard software error checking • Reload from non-volatile memory • System watchdog
Power failure recovery	• Establish redundant memory • Automated recovery routines
Compromised operating system	• Code redundancy
User	
Inadvertent misuse	• Design of bioreactors and instrumentation anticipate and preclude misuse • Failsafe operation • Clear instructions for use • Field maintenance program • Remote service link-up
Inadequate operational records	• Process tracking and storage in non-volatile memory • Data output via computer link • Compliant with regulations on electronic records
Unauthorized use	• User security codes/redundancy

3.3.3. Commercial challenges

Whereas the promise of tissue engineering has captivated many enthusiasts and has generated significant international investment in research, commercially engineered products are confronted with tough economic realities related to uncertain cost–benefit performance and ultimate eligibility for reimbursement. The limited and perhaps discouraging commercial progress to date could be related to the fact that the basic procedures for generating engineered tissues have generally been based on conventional manual bench-top cell and tissue culture techniques.

These manual techniques seem particularly appealing during the initial stages of product development (particularly for start-up initiatives) because the simple and widespread manual approach is generally viewed as a route to minimize initial development time and investment costs for rapid entry into clinical trials and the marketplace. However, as scientific, technical and commercial momentum builds, production costs associated with manual production quickly become a barrier. In addition, traditional techniques have inherent contamination risks and intra-/ inter-operator variability.

Furthermore, pragmatic limits to scale-up of unit production volumes and related inefficiencies in traceability pose significant challenges to business models based on manual protocols. As an alternative, a closed, standardized and operator-independent bioreactor-based production system has great benefits in terms of safety and regulatory compliance. Despite initially incurring high product development costs, this approach has great potential to improve the cost-effectiveness of a manufacturing process in the long run and maximize process scale-up (Table 19).

The advantages of a bioreactor-based approach appear convincing and yet bioreactor technology is not widely adopted. This cannot be because of a lack of competent bioreactor designs; a literature or patent search reveals a plethora of various bioreactors developed to date. So why has the drive to implement

bioreactors for the manufacture of engineered products for clinical applications been limited? Perhaps the clinical vision in different areas of tissue engineering is still relatively immature and hence quality, validation and productivity demands are considered secondary challenges.

One factor contributing to the delayed introduction of novel bioreactor technology could be the change in mindset required to break from well-established cell culture methods. To make this leap and for bioreactors to gain greater general acceptance, simple and more user-friendly bioreactor systems need to be introduced during the research phase, given that the routine handling of complex and technically challenging systems may discourage their introduction into laboratories that lack a specialized and trained workforce.

Moreover, even if it is acknowledged that bioreactors can generate engineered tissues of higher quality, there are currently few data highlighting the process reproducibility and – to the best of our knowledge – no sound analysis that critically assesses the potential cost-effectiveness of a bioreactor-based production system. Indeed, economic tools to guide investment decisions and to evaluate the potential cost-effectiveness of an engineered product are complex, rely on strong assumptions and are not yet well established.

As a first step, typical methods for the analysis of factors influencing strategic planning need to be applied to the commercialization of bioreactor-based tissue engineering for the clinic.

Without reference to a specific clinical indication, the results of a SWOT (strengths, weaknesses, opportunities and threats) analysis (Table 21) indicate that bioreactors for the clinical delivery of tissue engineering have attractive strengths and opportunities.

Not surprisingly, the new bioreactor-based approach also has certain weaknesses and poses threats that need to be addressed as part of a well-developed clinical and regulatory plan. To provide more quantitative indications, the headroom method has been proposed as a simple but rigorous approach to obtain preliminary conclusions on the cost-effectiveness of a new tissue engineering treatment (254).

Difficulties in implementing such analytical tools are related to the fact that some critical parameters required as input, for example the expected improvement in clinical performance, can only be arbitrarily assumed because of the uncertainties associated with an undeveloped technology.

The lack of cost–benefit analyses in the context of clinical efficacy precludes a plausible assessment of the likelihood of reimbursement coverage for the production and surgical implantation of engineered products, which in turn severely impacts the credibility of commercial business plans founded on the widespread use of tissue engineering. Given the problems with the tissue engineering business model and the variable demand for bioreactor systems, it is not surprising that industry has been hesitant to embrace commercialization of engineered products and invest significant resources to develop and/or use sophisticated manufacturing technology.

Closer interactions among scientists and engineers and stakeholders involved (regulatory bodies, clinicians, industrial partners, healthcare providers) are required for integrated bioreactor designs that can be used in systems that are practical and economical for the clinic.

Data from nearly two decades of bioreactor technology for engineered tissues are available. Although these research-oriented systems are generally too complex, user-unfriendly, unsafe, and expensive for direct use

in clinical applications, their underlying principles could nevertheless lay a solid foundation for more clinically compliant manufacturing systems.

Table 21: Analysis of bioreactor-based manufacture of tissue-engineered (TE) products.

Strengths	**Weaknesses**
• Bioreactors enable consistent implementation of bioprocesses under regulated culture conditions to maximize TE productivity • Smart bioreactor systems using integrated sensors provide a foundation for validation of TE processes for implant production • Intelligent system operation enables autonomous sequences to be delivered without the need for continual staff intervention • Bioreactor control systems use protective measures to ensure the process output is safe and effective • The automated system and related user interface controls are easy to use within implant production facilities • Pre-configured bioreactors and related system components provide low-cost routine operation following initial set-up • Automatic monitoring, collection and archiving of process data enhance institutional compliance with regulatory requirements • TE production systems based on automated bioreactor • technology are ideally suited to progressive scale-up of process throughput	• No pre-existing market model to use as a commercialization benchmark • Operational constraints associated with bioreactor design potentially limit visualization of an ongoing process • Automation requires initial investment in capital equipment; however, follow-on costs are economical
Opportunities	**Threats**
• Large regenerative medicine market (241) provides opportunity for TE-based product derivatives once effective implant production and delivery methods are available • Success of a few representative models of bioreactor-based delivery of TE into the clinic will accelerate commercial and healthcare provider interest • Technical and administrative consistency of bioreactor-based TE supports transition and expansion from research through to the clinic • Adoption of bioreactor-based standards will enhance clinical data analysis and reinforce regulatory documentation • Inherent consistency and quality of bioreactor output maximize the opportunity to pursue effective reimbursement for TE procedures • Continued refinement of scientific insights into tissue engineering processes can be translated to the clinic via bioreactor upgrades • Early focus for TE in clinical therapeutics is likely to be on critical conditions that ensure recovery of bioreactor R&D costs	• Cost–benefit of cell-based products might be insufficient to compete with off-the-shelf alternatives • Use of preconfigured bioreactor assemblies and automated techniques might require a change in mindset for certain users • Operation of a system to produce cell-based implants via automated techniques potentially displaces specialized workforces • Underlying science of bioreactor-based TE might be limited by insufficient supporting data (e.g. mechanism of action) • Innovative bioreactor technology might be constrained or delayed by mismatch with established regulatory standards • Implementation of bioreactor-based TE strategies could be constrained or delayed by validation challenges inherent in the adoption of automated processes (e.g. lack of appropriate quality control markers)

This will require the identification of only the most essential processes, culture parameters, and construct parameters that must be monitored and controlled to standardize production and provide meaningful quality and traceability data, at the same time as minimizing risks, costs, and user complexity.

In this context it is questionable whether complex and rather costly automated/robotic systems, which essentially mimic established manual procedures, can actually demonstrate real cost–benefit by replacing manual cell culture techniques in a manufacturing process.

Moreover, current automation techniques might fail to capture the expert nature and role of the cell culture technician. Instead, bioreactor designs could be dramatically simplified and related development costs significantly reduced if we re-evaluate the conventional tissue engineering paradigms and implement novel concepts and techniques that could streamline the individual bioprocessing steps.

Simplified tissue engineering processes could be the key to future manufacturing strategies by requiring only a minimal number of bioprocesses and unit operations, thereby facilitating simplified and compact bioreactor designs with limited automation requirements, with the likely result of lower product development and operating costs.

Here we underline the importance of simplicity and minimal costs while maintaining the core attributes of implant consistency and overall manufacturing productivity and scalability. Although we have attempted to draw several parallels to bioreactors in other fields of biotechnology, the analogy begins to break down during up-scaling, especially in the context of autologous implants.

Given that cells from each patient are highly variable and must be processed as completely independent batches, we cannot simply scale up the total volume as for a fermentation process or even an allogeneic product. Instead the production of autologous products must be ‘scaled out’ (i.e., a large number of parallel bioreactor systems independently performing the same bioprocesses). While scaling-out will require a high level of process reproducibility among each of the bioreactors, production could be readily up-scaled by simply adding additional (low-cost) units as product demand increases (255).

Finally, we must ask where the potential exploitation of bioreactors lies within the broad range of tissue engineering applications. Could there be a market for a simple and user-friendly bioreactor for general use in basic research applications? Are hospitals a viable market? A closed GMP-in-a box system would allow competent hospitals to manufacture engineered grafts on site, without reliance on a centralized industrial manufacturing facility.

Another consideration is whether the predominant market would comprise industrial firms producing engineered products in-house, with the perspective of introducing automated bioreactors within their manufacturing process. At present, none of these markets can be excluded and it is likely that business models will have to be adapted to some specific initial decisions. For example, it is possible that a centralized manufacturing facility is appropriate for the engineering of allogeneic grafts, whereas hospital-based production could become a reality for autologous cell-based tissues.

3.3.4. Bioreactor development roadmap

Bioreactor-based procedures for the translation of tissue engineering strategies into clinical products offer opportunities for exploitation and wide implementation of cell-based grafts as therapeutic solutions. But several challenges need to be met in order to make this paradigm successful, as outlined in the roadmap

proposed in Fig. 151. Bioreactor-based manufacturing concepts have to be introduced as soon as possible in the development of engineered tissue products.

This will allow industries to address underlying research questions (e.g. testing the effect bioreactor culture by parametric studies), carry out pre-clinical studies (e.g. testing the *in vivo* performance of tissues with consistent features) and keep strong ties with regulatory and commercial aspects (e.g. compliance with safety requirements).

In the proposed roadmap, scientific, regulatory and commercial aspects should be considered as a whole in each of the translational levels from initial idea to final implementation of clinical products, leading to continuous refinements and corrections.

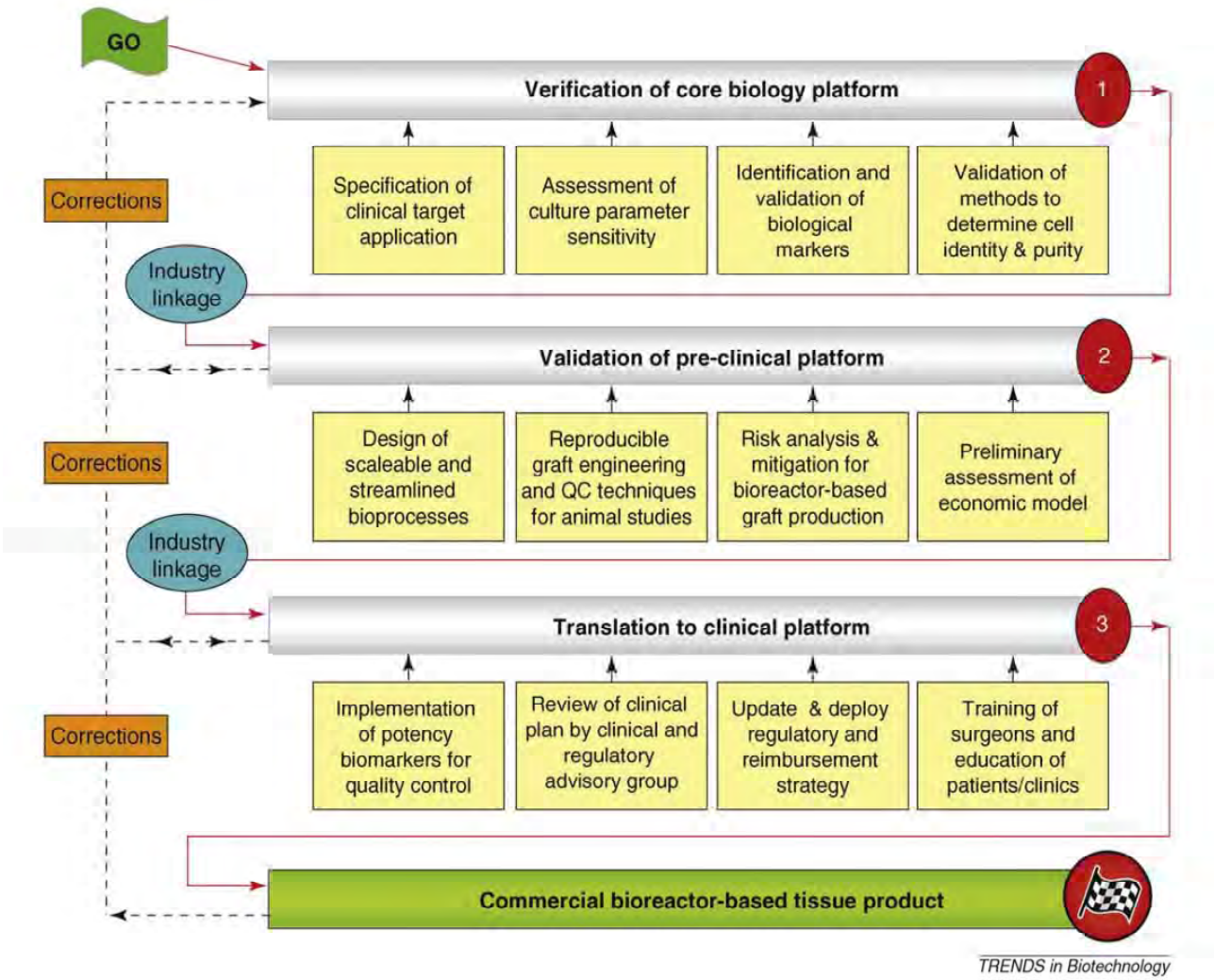


Fig. 151: Proposed roadmap for translating bioreactor-based engineered products into the clinic. Highlighted are the most critical scientific, regulatory, and commercial challenges that will need to be addressed, irrespective of the particular tissue engineering application. On starting to develop a bioreactor-based tissue engineering system and targeting a specific clinical application with clear goals in terms of projected clinical outcome, core biological criteria associated with the engineered product, the relevant bioprocesses and basic regulatory guidelines must be established and verified. Planning and conducting sound pre-clinical studies will allow assessment and validation of the criteria previously established for the core biology platform, most likely leading to refinement and optimization of the bioprocesses and to a better understanding of the underlying biology. Interactions with industrial partners will facilitate the transition between platforms through the evolution and industrialization of research-based technologies and by defining the commercial potential of the product. In the final stage of the roadmap, which clearly is the most challenging and critical, translation to the clinic platform will require clinical, regulatory and reimbursement strategies (256)**.**

Finally, the success of bioreactor-based tissue engineering products will depend on acceptance of the proposed strategies by the stakeholders involved (e.g. surgeons and industry alliance partners (257)) and by the general public (i.e. the patients) (258). In fact, while the availability of therapeutic products has the potential to develop the market, key for their ultimate success will depend on the generation of a receptive society, which needs to be educated by means of proper dissemination activities.

4. Development of membranes for oral implantology

Provided the studies reported in Chapter 1 and the regulatory advantage of developing dental products, as stated in paragraph 3.2, a strategy aimed at developing membranes for oral implantology was designed. These membranes are used when dental implantation is seriously hampered by either lack of sufficient bone volume or by periodontal disease.

4.1. State of the art of Guided Bone Regeneration (GBR) and membranes for oral implantology

Since the significant results of studies by various authors such as Brånemark (259), (260) and Schroeder (261), the use of dental implants has become a universally accepted treatment for the replacement of missing elements in patients partially or totally edentulous, with a high percentage of success of about 85% and above after 5 years. Limitations in the use of this methods result from the need to have alveolar crests with enough bone volume to receive the implants placed in a submerged position, i.e. endosseous. Over the last few years clinical and experimental studies have concentrated on the use of guided bone regeneration (GBR), i.e. a technique of reconstructive surgery that also envisages extending indications for the use of osseointegrated dental implants to patients with unfavourable anatomic conditions. Making us of previous experiences deriving from orthopaedics and periodontology (262) (263) (264) (265), attempts were made to resolve cases in which the ideal prosthetic position of the implant is located in an area lacking the amount of necessary bone to insert the dental implant.

Thanks to the development of the GBR procedure, those particular situations in which a peri-implant osseous defect remains after the insertion of the fixture are no longer considered negative, as the growth of newly formed bone can fill these gaps.

There are several different technical solutions for a clinical application of the principles of GBR, such as the utilisation of both resorbable and non-resorbable membranes, autologous and heterologous bone grafts as well as alloplastic filling materials.

The principle of physically isolating an anatomical site in order to stimulate healing of a particular type of tissue through the use of mechanical barriers has already been exploited since the 1950s by Hurley (266) and by Murray (267) in orthopaedic surgery. They had established the fundamental conditions for obtaining correct bone regeneration: (i) presence of a blood clot; (ii) contact of the blood clot with vital tissues and (iii) preservation of osteoblasts.

On the other hand, guided bone regeneration (GBR) is based on three fundamental requirements (i) cellular exclusion to avoid the invasion of epithelial and connective tissue that grows at the defect side instead of bone tissue; (ii) mechanical stability to protect and allow healing process; and (iii) space maintenance to guarantee space into which cells from the surrounding bone can migrate (268).

Results expected from regenerative therapy include: (i) decrease in probing depth; (ii) improvement in the clinical level of attachment and (iii) contraction of marginal tissue. The encouraging results obtained, regardless of the different techniques used (non-resorbable and resorbable membranes on one hand and, on the other, bone grafts or filling materials or even possible combinations of the two procedures) have led to the use of this method in the field of osseointegration for those special conditions of insertion of implants with residual peri-implant osseous defects such as dehiscences, fenestrations, intraosseous defects or, more recently, for cases in which the presence of insufficient crest height is observed.

An ‘‘ideal” membrane should be biocompatible, non-immunogenic, non-toxic, bioabsorbable with a degradation time comparable to achieve bone regeneration before membrane disintegration. Various

commercially products, ranging from non-resorbable materials to resorbable membranes, are available. Non-resorbable membranes retain their build and form in the tissues, requiring a second surgical procedure for removal, increasing patient discomfort, as well as the costs and duration of therapy (269). Non-resorbable membranes include: (i) expanded polytetrafluoroethylene (e-PTFE, Gore-Tex®), (ii) high-density polytetrafluoroethylene (d-PTFE) and (iii) titanium reinforced expanded polytetrafluoroethylene (Ti-e-PTFE) membranes. In detail, the e-PTFE involves a microstructure consisting of solid nodes interconnected by fine, highly oriented fibrils, providing a unique porous structures. The expanded PTFE is neither woven or knit and thus will not fray nor is abrasive and consists of two parts: (i) an open microstructure collar (90% porous) which promotes connective tissue ingrowth, positioned coronally, able to inhibit or retard the apical migration of epithelium during the early phase of wound healing, and (ii) an occlusive membrane (30% porous), serving as a space provider for regeneration and as a barrier towards the gingival flap (270). Different drawbacks are recognized, such as (i) the need for a second surgical procedure, possibly causing undesirable bone resorption and (ii) their stiffness may result, in a high percentage of cases, in dehiscence of the soft tissues with exposure of the membrane and bacterial contamination (271). An alternative to e-PTFE is a high-density polytetrafluoroethylene (d-PTFE) membrane (Cytoplast Regentex GBR-200 and TXT-200; TefGen-FD), characterized by submicron (0.2 μm) porosity size. It is easy to remove, avoiding the need for a difficult second surgery and is already exposed and visible at the surgical site. No local anaesthetic or flap dissection are required (272). Titanium-reinforced e-PTFE membranes (Cytoplast® TI-250; TR9W Gore-Tex) are also available. The addition of titanium strut provides support to the overlying soft tissue preventing its collapse into the defect, resulting in increased bone volume. Additionally, in a minimally invasive technique, the presence of the strut allows the surgeon to precisely position the membrane under flaps with minimal dissection and flap reflection (273). On the other hand, various resorbable membranes are currently available as alternatives to conventional non-resorbable membranes and have been highlighted because they permit a single-stage procedure, reduce patients' discomfort and costs and eliminate potential surgical complications (274). Because of their biodegradation, resorbable membranes elicit tissue reactions which potentially influence wound healing and compromise regenerative outcome. Moreover, the importance of the ability to create sufficient space underneath the membrane has been established, and a resorbable membrane should remain stiff long enough to maintain the required space for bone regeneration. Resorbable membranes should be biocompatible and the infection risk to partly exposed membranes should be minimal (275). In general, the commercially available GBR bioresorbable membranes are made of polymers, including bioabsorbable polylactide (PLA), polyglycolide (PGA), poly(ε–caprolactone) and their copolymers and collagen. Commercial available products are: (i) Guidor® Matrix Barrier, composed of polylactic acid softened with citric acid ester to achieve malleability to ensure close adaptation of the barrier to the bone surfaces surrounding the defect. It consists of a double-layered matrix with two differently perforated layers: (a) the external layer, designed to allow integration of the overlying gingival flap, has large rectangular perforations to promote tissue integration and to enable gingival connective tissue to penetrate quickly into the matrix and (b) the internal layer has many minute circular perforations able to retard tissue penetration but still allow for nutrition. According to the manufacturer the degradation by hydrolysis of the material starts shortly after implantation, but does not influence the macrostructure of the barrier for at least the first 6 weeks (276), (277). (ii) Resolut® (W.L. Gore & Assoc), consisted of an occlusive membrane of glycolide and lactic copolymer and a porous web of polyglycolide fibers. The occlusive membrane prevents cell ingrowth, and porous part promotes tissue integration (278); (iii) Epi-Guide® Bioresorbable Barrier Matrix is a membrane designed for use as an adjunct to periodontal restorative surgeries and assists in the regeneration of bone and periodontal support tissues. It is a porous, three-dimensional matrix fabricated from D, D-L, L polylactic acid. The barrier's three-layer construction is designed to maintain its architecture and structural integrity for 20 weeks after implantation with complete

bioresorption between 6-12 months (279). Collagen membranes are also applied to the GBR technique due to their low antigenicity, high biocompatibility, excellent cell affinity and controlled biodegradability induced by cross-linking reagents (280). Drawbacks of collagen membranes are: (i) the loss of its space-maintaining ability in humid conditions and (ii) the implantation of animal derived collagen includes a potential risk of disease transmission from animal to human (281). The most important commercial membrane is Bio-Gide® (Geistlich Pharma AG), composed of porcine type I and type III collagen fibers without any organic components and/or chemicals, and has a bilayer structure composed of a ‘‘compact’’ and ‘‘porous’’ layer. The compact layer of the membrane possesses a smooth and condensed surface to protect against connective tissue infiltration, while the porous layer permits cellular invasion. The cited layers may enable osteogenic cell migration and prohibit connective tissue intrusion, respectively (282).

Table 22 summarizes some of the products on sale.

Table 22: Products on sale.

Trade name	Manufacturer	Composition	Notes
Bio-Gide®	Geistlich	Non-crosslinked collagen	Bioresorbable
BioSorb®	Imtec	Bovine collagen	Bioresorbable
BioCellectTM	Imtec	Lactic acid homopolymer	Bioresorbable
BioBarrierTM	Imtec	PTFE	Permanent (requires surgical removal)
BioMend®	Zimmer	Bovine collagen	Bioresorbable
T-Barrier-1®	B&B Dental	Equine collagen	Bioresorbable in 80/90 days
T-Barrier-2®	B&B Dental	PTFE	Permanent (requires surgical removal)
T-Barrier-3®	B&B Dental	Titanium	Permanent (requires surgical removal)
Tutodent® membran	Tutogen – RTI Biologics (Zimmer)	Equine collagen	Bioresorbable

4.2. Fund raising strategy

The fund raising strategy was based on two opportunities, one given by Italian Ministry of Economic Development and one by the European Community. So, two development projects where tailored to those Calls:

- B^3-BARRIER (Dispositivi bioartificiali e bioriassorbibili con proprietà biomimetiche e di barriera selettiva per la chirurgia rigenerativa orale), in the ambit of “ACCORDO-QUADRO MI.S.E.-ICE-CRUI

Attuazione 2010" Italian Ministry of Economic Development, that aims at developing bioresorbable functionalized membranes for oral implantology;

- BIO-PATH (BIOengineered barrier membranes for the treatment of dental bone PATHologies), in the ambit of "MANUNET TRANSNATIONAL CALL 2011", that aims at developing resorbable and non resorbable functionalized membranes.

Being fundamental their technical feasibility and economic affordability, these projects have been written keeping in mind the importance of IPR protection and the economic impact they bring.

They are described in the following paragraphs.

4.3. B^3-Barrier

4.3.1. Project partners' list

- Laboratori Biomicron s.r.l. (BIOMICRON)
- Politecnico di Torino (POLITO)
- Hacettepe University of Ankara, Turkey - Institute for Pure and Applied Sciences (UNIANK)
- Bioindustry Park Silvano Fumero/bioPmed (BIOPARK)

4.3.2. Project objective

The project B^3-BARRIER aims at producing innovative barrier devices that can be applied in periodontology and in advanced regenerative surgery, with resorption times compatible with those necessary for bone regeneration. The devices will be composed of three bioresorbable overlapping layers, each with different physicochemical and morphological characteristics suitable for the specific regenerative function, capable overall of satisfying the needs of dental surgery. The proposed device will be optimised to satisfy the three fundamental biological principles of guided bone regeneration: (i) cell exclusion, (ii) stabilisation of the wound and (iii) maintainment of the space

The barrier device will be made of one dense intermediate layer and two porous layers. The dense layer of the device, made of biocompatible and bioresorbable synthetic polymers, will prevent the cell passage of gingival fibroblasts that may interfere with the bone regeneration process and will provide the mechanical stability and manageability required by the entire device. Another objective of the project will be to optimise the composition of the non-porous layer through a blend of different bioresorbable polymers so as to modulate their resorption time and limit the inflammatory process during resorption.

The porous layers of the membrane made of a mixture of natural and synthetic polymers (bioartificial blends) and, in one case, mixed with hydroxyapatite for the layer that will foment bone regeneration, will be designed with suitable chemicophysical and morphological characteristics to favour tissue regeneration at contact. More specifically, this layer will be made using the electrospinning procedure in the form of nanofibrous substrate, modulating the dimensions of the fibres and pores, the degree of porosity and composition, in order to favour adhesion, proliferation, cell growth and migration (osteoblasts for the layer in contact with the bone and gingival fibroblasts for the external layer). In particular, the composition of the porous layer that will expedite wound healing will include natural polymers with anti-microbial properties capable of fomenting cell adhesion.

Overall, the devices will provide a biological seal that, in association with primary stabilisation of the wound, will enhance tissue regeneration. More precisely, the membranes will allow: (i) appropriate guided bone regeneration; (ii) hemostatic, chemotactic, angiogenetic and osteoblastic properties that will favour

tissue regeneration; (iii) total resorption in 6 to 9 months; (iv) perfect biocompatibility; (v) an anti-inflammatory, eutrophic and cicatrizant capability; (vi) anti-bacterial properties and (vii) simple application on the site of the defect.

The B^3-Barrier project aims at producing innovative barrier devices and seeks to implement a simple and fast production process from economical materials if possible that are designed for future industrialisation of the product and competitive prices.

Development of the multilayer device

The initial phases of the production process will consist in making the three single layers separately, by means of electrospinning techniques for the two porous external layers and compression moulding for the device's dense internal layer. Subsequently, the two membranes will be assembled into a single device, selecting the most suitable technique among (i) a vacuum heat treatment or (ii) under pressure (that consists of applying the dense layer heated to a temperature that is sufficient to make it plastically deformable, between the two porous layers due to the effect of the vacuum or mechanical pressure) and (iii) the use of a suitable chemical agent to bind the layers of the device. Adhesion among the membranes of the device will be optimised through a suitable selection of assembling process parameters: time, temperature and pressure/vacuum applied in one case; change of the surfaces of the two layers in contact (for example, by plasma treatment) and selection of a suitable adhesive chemical agent (for example, polyethylene glycol diacrylate, PEGDA) in the second case. The assembling method of the layers selected will be the one that allows a high degree of adhesion without significantly changing the morphological characteristics of the nanofibrous layers.

More specifically, the dense layer will be made of a binary polymer blend of bioresorbable and biocompatible polyesters, such as polycaprolactone (PCL), polyglycolic acid (PGA), polylactic acid (PLA) and their copolymers, and will provide the necessary barrier function by protecting the newly-formed bone from the infiltration of fibrous connective tissue of soft tissues. The composition of the polymeric blend will be optimised to obtain membranes with mechanical and degradation/bioresorbability properties suitable for application (optimisation phase of bulk characteristics and limited inflammatory response). The dense layer will be prepared with the compression moulding technique, a simple and economical method, and will have a thickness of 100-120 µm, corresponding to about one-half of the standard thickness of the devices for guided bone regeneration currently on the market.

The two porous layers will be membranes (each with a thickness of 50-70 µm) made of a network of nanofibres obtained by means of the electrospinning technique. The dimensions of the pores and degree of porosity will be optimised in order to enable cell colonisation of the porous matrix. Nanofibres will be made of blends of natural and synthetic polymers (bioartificial blends) or will be combinations of nanofibres blended with natural and synthetic polymers (bioartificial nanofibres) (Fig. 152).

The use of bioartificial polymeric materials, in general, allows us to integrate the good mechanical properties of synthetic polymers with the good properties of biodegradability and biocompatibility of natural polymers (283), (284). The bioartificial materials used in the porous layers will allow us to obtain a better mechanical stability and times of degradation and bioresorption compatible with the formation of new bone tissue and healing of the surgical wound. More specifically, bioartificial materials of the external layers will be made of a biocompatible, bioresorbable synthetic polymer with a higher fusion temperature compared to the polymeric blend that constitutes the dense layer, in order to prevent the collapse of the nanofibrous structure during the assembly phase of the two layers by heat treatment. A possible choice for the synthetic polymer could be polylactic acid (PLA). Like natural polymer, the gelatin will be used for the

layer in contact with bone tissue and gelatin and chitosan for the layer in contact with epithelial and connective tissue. Gelatin is a protein derivative that is obtained from collagen. It is biodegradable, bioresorbable in vivo and it is not antigenic under physiological conditions and its chemical and physical properties can be easily modulated through chemical modification (285), (286), (287). Compared to other membranes currently on the market and made of collagen, the use of gelatin has advantages from the economic point of view and as a process: gelatin is much cheaper than collagen and is also soluble in water at high concentrations. Many clinical and experimental studies can be found in literature that have demonstrated it use and success in tissue engineering and in bone tissue regeneration in particular (288), (289), (290). In order to solve the major problems regarding the use of gelatin for in vivo applications, represented by its solubility in aqueous solutions, it will be suitably crosslinked by enzyme catalysis (transglutiminase) and/or non-cytotoxic crosslinkers, such as for example carbodiimides and genipin.

Chitosan is a natural polysaccharide obtained from chitin by deacetylation. It is a low-cost, biodegradable, biocompatible, non-toxic material with bacteriostatic properties.

The composition of the porous layer in contact with epithelial and connective tissue will be optimised in order to enhance adhesion and cell proliferation and prevent bacterial colonisation.

For the layer designed to foment bone regeneration, an inorganic phase (hydroxyapatite, HA) will be introduced into the composition of nanofibres in order to render the porous layer biomimetic. In bone tissue engineering, the term biomimetic is understood to be a device marked by a composition similar to that of bone tissue. Recent studies have shown how biomimetic scaffolds improve adhesion and proliferation of osteoblasts, favouring excellent healing of the bone (291), (292), (293). Hydroxyapatite incorporated is also capable of absorbing cell adhesive proteins from the serum, such as fibronectin and vitronectin, further fostering cell growth (294). In conclusion, the incorporation of gelatin and hydroxyapatite in the porous layer in contact with the bone will foment the adhesion of osteoblasts mediated by peptide sequences of the gelatin and will confer osseoinductive and osseoconductive properties to the device. The basic nature of hydroxyapatite will also partly buffer the acidic degradation products of the bioartificial layer, with a reduction of the inflammatory response.

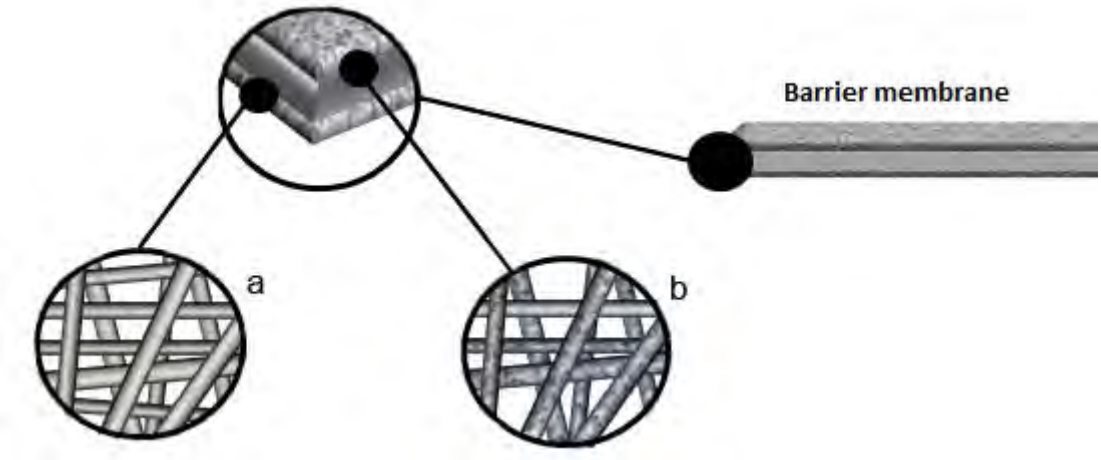


Fig. 152: Detail of the device's multilayer structure. In detail, nanofibrous porous layer in contact with a) gingival connective tissue and b) alveolar bone.

Innovativeness

This project aims to commence from the basis of current state of the art polymeric formulations for the preparation of barrier devices for regenerative dental surgery. Products currently marketed have

disadvantages that have yet to be overcome: resorption kinetics of the bioresorbable devices is too fast, while non-bioresorbable devices require second surgery for their removal, which may expose the implant site to bacterial attack, with the development of infections of bone tissue and interruption of the regenerative process.

The products of the B^3-BARRIER project define a new type of implantable device for regenerative oral surgery with several innovative aspects, such as:

- An innovative design, made of one dense layer to isolate the bone regeneration process and stimulate wound healing at the same time, and two nanofibrous external layers with characteristics suitable for bone regeneration and healing of the surgical wound. The combination of membranes obtained through the electrospinning technique with dense polymeric films in particular, has never been investigated for biomedical applications.
- The presence of two interfaces with specific characteristics for mediating the biological response with tissues in contact: one nanofibrous, porous layer mixed with hydroxyapatite with biomimetic properties for bone regeneration and one nanofibrous, porous layer with a composition appropriately selected to prevent bacterial infections and at the same time foment wound healing.
- The property of bioresorbability with resorption times modulated by bone regeneration time, thanks to the combination of various polymer blends in both the dense layer and in the porous layers of the device.
- The composition made of gelatin and chitosan, which enables a significant reduction in the cost of the final device, compared to bioresorbable devices currently on the market made of collagen, which require high processing and production costs.
- The proposed production process, which is simple, economic and easily industrializable.

4.3.3. Consortium detailed description

POLITECNICO DI TORINO (POLITO) – Department of Mechanics

The Polytechnic of Turin is in the lead in technological research backed by its historical context. At the Polytechnic, today's needs are analysed to plan a sustainable future. The Industrial Bioengineering group of the Department of Mechanics is a leader in bioartificial systems research in the field of bioactive molecular systems, biomolecular design, tissue engineering, biological fluid dynamics and research in the field of Medical Devices. The Bioengineering research group is present in the European technology platform NANOMEDICINE and participates in national, European and international research projects.

The Polytechnic of Turin will provide the project with the consolidated and internationally recognised experience of its researchers and efficiency of its laboratories and instruments in the field of applied chemistry in the biomaterials and tissue engineering sector.

As part of the project, the Polytechnic of Turin will use commercial polymers and solvents for producing polymeric matrices. The instruments available for research within the group are: chemical hoods, incubators, lyophilizers, dip-coaters, equipment for chemicophysical (FTIR, UV-Vis, DSC, TGA, instrumentation for contact angle measurement) and mechanical (traction, compression, fatigue, nano-indentometer tests) characterisation. Other instruments available at the Polytechnic of Turin for the processing of polymers are: mechanical presses, Brabender Plasticorder for blending polymers in the molten state and extruders. The Industrial Bioengineering group of the Department of Mechanics is composed of 2 full professors, 2 associate professors, 3 senior researchers, 4 research assistants and 8 doctorate students.

In particular Prof Gianluca Ciardelli will be involved in the project with expertise in the field of the design and production of polymeric materials for applications in the field of tissue engineering. Specific expertise for the project will be recluted by 2 temporary research associates. Expertise sought will be in the field of polymeric biomaterials and bionanotechnologies.

Laboratori Biomicron (BIOMICRON)

Established in 1978, Biomicron's mission is the development and production of single-use technical articles and biomedical analysis kits for the laboratory. It has been operating in this field for over thirty years in partnership with the sector's largest companies. Its clients include: Alk-Abello, Anallergo, DiaSorin, Halallergy, Lofarma laboratori, Sorin cardio and Sarm allergeni.

The first production line concerns microtiter plates with multiple "wells" for carrying out immunological techniques. Production is handled by a co-owned moulding company that manufactures the articles through the transformation by injection of selected tecnopolymers.

In the 1980s, it participated with the research and development sector in the optimisation of a storage container for heart valves in which the product's intrinsic properties are combined, such as functionality, protection and the possibility of sterilisation with ethylene oxide. The project became operational and expands from two types of mechanical valves to the biological valve. Biomicron also developed a medical device that optimised diagnostic capability in allergology that is still marketed. The company turned its attention to high performance liquid chromatography (HPLC) and, with Bioanswers, was involved in the research and production of HPLC kits. A patent for the determination of alcoholemia was developed in this sector.

As Bioanswers, it is also deals with bioreactors applied to industrial fermentation technologies, developing techniques, controlling production and the set up of controlled fermentation plants of leading companies.

As part of this project, the partner Laboratori Biomicron srl will supply consumable materials as well as its company's know-how for the development of an innovative product of great economic interest, reducing investment in design.

Hacettepe University of Ankara, Turkey (UNIANK) . Institute for Pure and Applied Sciences - Plasma Aided Bioengineering and Biotechnology Research Group (PABB)

Activity carried out by the Plasma Aided Bioengineering and Biotechnology group (PABB) at the University Hacettepe of Ankara is linked to the study of all problems concerning the conception and implementation of innovative solutions devoted to life sciences. The PABB group is specialised in the funtionalisation of materials through different technologies including plasma treatment. The group's scientific activity is currently involved in various areas such as Bioengineering, Nanomedicine, Nanotechnologies and Food engineering.

The PABB group will provide the project with its consolidated and internationally recognised experience in the field of treatments for the functionalisation of biomedical devices.

Prof Mehmet Mutlu is currently Full Professor at the Department of Nanomedicine and Nanotechnologies of Hacettepe University of Ankara. His expertise ranges from the field of Biotechnologies and Bioengineering to Nanobiotechnologies and Nanomedicine. During the device's production phase, Prof Mutlu will provide his knowledge of heat treatments and plasma surface modifications.

Bioindustry Park del Canavese SpA (BIOPARK)

It is a scientific and technological park devoted to life sciences located in the Piedmont Region. The Park operates in 4 main areas of activity: establishment of companies, start-up support, optimisation of the results of research and supply of scientific services, participation in targeted and special regional, national and international projects. In its activities the Park's overall objective is to foster the development of the biotechnology sector with particular attention to the creation of new companies and to aspects of of technological transfer. Recently the park has been indicated by the Piedmont Region as a cluster managing company of the biotechnology and biomedical innovation cluster bioPmed (www.biopmed.eu). The cluster officially includes over 63 public and private entities and has set itself the following objectives:

- identify operative synergies among adherents aimed at the implementation of common research projects;
- promote the regional system in a national and international context;
- supply and make available information and services;
- participate as a cluster in international projects.

Fabrizio Conicella is currently General Manger of the Bioindustry Park and in charge of Business development after having been the project coordinator from 1999. He has participated in various regional, national and international projects aimed at technological transfer activities and company development, as well as various internationalisation projects. He is Project Manager of various European projects and currently coordinates the bioPmed cluster. He is a member of various groups on issues regarding company development and technological transfer, a member of the board of directors of Assobiotec and managing board of the Council of European BioRegions.

Role in the project - Manager of the following output:

- Analysis of the means of exploitation of the project's scientific results;
- Scouting activity for additional potential companies that could become involved in the project;
- Support for promotional activities and contact with companies, in particular SMEs;
- Support for the analysis and the creation of national and international promotional/distribution channels of project results.

4.3.4. Expected results

B^3-BARRIER aims to produce an innovative biomedical, bioresorbable device in the form of a membrane for applications in advanced regenerative surgery, in particular in guided bone regeneration in dentistry. The product will have a differentiated structure and composition of the two interfaces with alveolar bone and gingival connective tissue, so as to specifically foment bone and soft gingival tissue regeneration respectively. The central portion of the barrier device will have a dense structure in order to ensure a "barrier effect", i.e. prevent the infiltration of the epithelial and connective tissue (non-osteogenic cells) in the site of the bone defect, isolating the two distinct tissue regeneration processes on each implant/bone interface.

In more detail, the device produced will satisfy the requirements of:

- Biocompatibility;
- Controlled bioresorbability in 9-12 months;
- Clinical manageability;

- Space-making capability;
- Efficacy over time of the barrier effect;
- Anti-microbial activity (bacteriostatic properties);
- Mechanical, structural and functional integrity for a sufficient time for bone regeneration;
- Guided regeneration of bone and gingival connective tissue;
- Low production cost linked to the low cost of constituent materials and development of a simple and economical production process;
- Minimal environmental impact of the production process with limited use of potentially toxic organic solvents.

The product will perform better compared to barrier devices currently on the market (Table 1), while maintaining production costs substantially unchanged.

The product will be developed during the five phases of the B^3-BARRIER project. Results expected from each work phase are given below.

The device's main sector of application will be clinical dental medicine, to allow the insertion of permanent dental implants in cases of insufficient bone volume. Edentulism is an extremely important social problem: the National Institute of Dental Research has found that, for example, 42% of the population in the United States over 65 years of age and 4% between 34 and 64 years of age present a partial or complete absence of natural teeth and require restoration of masticatory function by means of permanent or removable prostheses. The traditional type of removable prosthesis is often poorly tolerated for both psychological and functional reasons due to the extreme difficulty in making a satisfactory product especially in terms of stability. The use of dental implants can resolve or improve these problems, allowing the assembly of a permanent prosthesis in place of a removable one in some cases and providing stability to removable prostheses in others. Data regarding the trend of the global dental market indicates a stagnation in some sectors of dental treatment in terms of market growth, whereas there has been almost an opposite trend in the rapid progress of implantology over the last few years. The most significant growth occurred in the Japanese, followed by the American and lastly the European market.

Italy is one the leading world markets with about 1 million dental implants applied every year and about 65% of Italian dentists carry out this type of intervention. The demand for dental implants in Italy is rapidly growing: according to research commissioned by the Italian Society of Osteointegrated Implantology (SIO). In a sampling of over 600 people, 68% requested the use of implants, considered more practical, durable and efficient compared to dentures. The respective global market value is estimated at 1.1 billion dollars and is growing 15% every year.

Moreover, the percentage of success of implants carried out after placement of suitable barrier membranes has reached 85% overall after one year and 80% 5 years after the intervention. There has therefore been an enormous expansion in the number of membranes for guided bone regeneration on the market, in the number of operators using them and clinical cases performed. The attention of clinics and manufacturers has turned to the identification of the causes responsible for the share of residual failures in order to remediate them. Therefore, "in vivo" and "in vitro" studies and experiments have increased in recent years.

In this context, the barrier device developed during the B3-BARREIR project will have e twofold economic and social impact. The B^3-BARRIER project will introduce innovative solutions and technologies for the production of multilayer barrier membranes for regenerative oral surgery, overcoming the limits of

products currently on the market. The barrier devices produced will be highly competitive due to their improved performance and low cost. The project will also provide finalisation of the industrial process for the production of the devices through the use of economical solutions with minimal environmental impact. The device will be released on the market after appropriate clinical tests and obtainment of the required certifications, expected within 3 to 5 years after the project's completion. The device is expected to have a strong economic impact on the global biomedical devices market, benefiting European SMEs that have been suffering recently from the competition of developing third-world countries.

The product developed will also provide better performance, which could lead to a social benefit in terms of reduction of morbility, surgical stress, risk of exposure and complications and possible failures.

During the B^3-BARRIER project, POLITO will make available its experience in the sector of biomaterials and biomedical devices in order to initiate a partnership between national and international research centres and an SME aimed at the development of a product intended for future marketing. It should be pointed out that the type of design of the device created, which assembles nanofibrous membranes on a polymeric base, is a very innovative aspect that could also find a use in other biomedical applications in the future through the optimisation of chemicophysical and mechanical characteristics for the specific application.

Therefore, the expected fallout from participation in the POLITO project will be:

- acquisition of new skills through training of staff in the field of devices for dental surgery;
- portfolio consolidation in the field of biomedical research;
- technological transfer in the industrial sector and consolidation of a ten-year cooperation with other research institutes and industries;
- intellectual property of the results obtained;
- international visibility following publications in an innovative sector;

On the other hand, the project will enable the financial participation of BIOMICRON in the development of an innovative product of great economic interest with reduced investment at the design stage.

The partnership between national industry and the Italian and Turkish research community (UNIANK) will further stimulate the development of solutions to meet demands in new areas of application. The cohesion between industry and academic research fomented by the project will help overcome difficulties encountered in Europe in transforming scientific and technological findings into economical and marketable industrial products, providing SMEs with technological solutions for their specific problems.

The partnership developed between BIOMICRON, POLITO, BIOPARK and UNIANK will provide the basis for further cooperation in the future aimed at narrowing the gap between industrial needs and the research community.

Thanks in fact to the support of BIOPARK, the project will make it possible to:

- assess additional future approaches to foment cooperation between R&D and companies;
- develop activities with regard to the exploitation of results, such as the opportunity for technological transfer that could emerge from SMEs to foster "fallout" onto an increasing number of both national and foreign companies.

It should be pointed out that the BIOPARK scientific park is also coordinator of the bioPmed innovation cluster (www.biopmed.eu) that brings together 63 players (September 2010) including 53 companies. This

situation ensures a privileged channel to reach out to not only local, but also international sectors of industry and application, thanks to the exploitation of the network of relations of the Park and the bioPmed cluster in particular through the CEBR (Council of European Bioregions - www.cebr.net) of which the Park is a member.

The European dimension of the project's impact will be strengthened by a distribution network that includes various industrial associations, such as EuroBio (European Association of Bio-Industries; http://www.europabio.org), and contact with COST actions (for example, the COST868 action "Biotechnological Functionalisation of Renewable Materials" that includes the participation of POLITO, UNIANK and other European research centres; http://www.cost868.tugraz.at/).

4.3.5. Project statistics

The detailed description of work and the GANTT diagram are not reported here. Nevertheless, the B^3-BARRIER project involves 32 person months; Table 23 and Table 24 show the total cost and financial coverage plan of the project. 50% of the project will be financed by Italian Ministry of Economic Development.

Table 23: Detail of B3-BARRIER costs.

Annual costs (sustained exclusively by the University proposing the Project)	
Internal human resources of the Italian university: Full Professor No. 1 x No. 4 months Associate Professor No. 1 x No. 4 months	€60,000
Recruitment of part-time assistants: Research assistant No. 1 x No. 18 months Research assistant No. 1 x No. 6 months	€42,000
Board, lodging and travel expenses	€5,000
Purchase of consumables (including company financing for €60,000)	€70,000
Leasing of instruments, laboratory, computer and audiovisual equipment, etc. *(indicate)*	€40,000
External consultations excluding those for preparation or coordination of the project	€20,000
Participation in conferences/seminars pertaining to project activities	€3,000
Total cost of the Project	**€240,000**

Table 24: Financial coverage plan of B3-BARRIER project.

FINANCIAL COVERAGE PLAN				
SOURCES		**INTERNAL HUMAN RESOURCES**	**FINANCIAL RESOURCES**	**TOTAL**
FINANCING ITALIAN UNIVERSITY (50%)		60.000		60.000
	Italian Entrepreneur Partners		60.000	60.000
PUBLIC CO-FINANCING (50%)	**ICE**		120.000	120.000
	Other public administrations:			0
		60.000	180.000	240.000

4.4. BIO-PATH

4.4.1. Project partners' list

- Laboratori Biomicron s.r.l. (BIOMICRON)
- Reiner Microtek SL (REINER)
- Politecnico di Torino (POLITO)

- Consorzio Proplast (PROPLAST)
- TEKNIKER

4.4.2. Project detailed background

Periodontal disease is a chronic bacterial infection characterized by persistent inflammation, connective tissue breakdown and alveolar bone destruction. The chronic inflammation associated with periodontal disease represents the host response to bacterial plaque, mediated by the environment in which the response occurs. The development of periodontal disease is affected by individual conditions, such as life-style and genetics. It often starts in middle age and its incidence progresses with age and is accelerated by systemic disorders such as diabetes, osteoporosis, and metabolic deficiencies. Guided bone regeneration (GBR) method is a well-established therapy to repair mandible and alveolar bone defects affected by periodontal disease, using membranes that prevent the invasion of non-functional scar tissue and encourage bone re-growth, allowing the subsequent application of osteointegrated bone implants. The current demographic trend of the European society for progressive ageing related to the increased life-span of the population is a factor stimulating the rapid development of GBR market niche, with increasing demand for high performance materials and technologies for GBR membrane production. In 2010, the total European markets for dental bone graft substitutes and other biomaterials grew by 4.4% over 2009 and were valued at nearly €232 million (iData Research, Inc. –"European Markets for Dental Bone Graft Substitutes, Dental Membranes and Tissue Engineering 2011 - Executive summary"- 2010). Conventional materials of GBR membranes are non-degradable expanded—polytetrafloroethylene (ePTFE: Gore-Tex®) (295) and degradable polylactic acid (Guidor®) (296) and polyglactin (Vicryl®) (297). Although PTFE membranes have achieved excellent clinical results, second surgery procedure is required to remove the membranes after new bone generation. In addition, they do not possess chemical cues to promote bone repair. On the other hand, degradable GBR membranes avoid second surgery which lightens the patients' burden. However, there are still challenges with respect to (i) barrier of tissue invasion associated with rapid degradation of membranes, (ii) osteoconductivity to achieve large area repair and (iii) mechanical stability of membrane to sustain surgery treatment.

The European biomedical SMEs are facing the challenge to maximise their opportunities for market competitiveness adopting suitable technologies for developing and commercialising innovative MEDTECH products. BIO-PATH project is aimed at the design of advanced MEDTECH products for GBR. In a more traditional approach, non-degradable PTFE membranes will be surface modified by environmentally friendly techniques, such as plasma treatment, followed by layer-by-layer spray coating, to tailor the surface properties, allowing rapid bone regrowth on the membrane side in contact with bone tissue and rapid wound healing on the side in contact with connective tissue. In a highly innovative approach, bi-layered bio-absorbable membranes will be developed, by the assembly of a compact and a porous layer. The not-porous layer will be based on biocompatible synthetic polymers and will confer mechanical strength and flexibility to the whole device. The physicochemical and structural features of the porous layer will be modulated by its chemical composition and the processing conditions for layer production, with the aim to promote bone repair. The porous layer, composed of either natural polymers or a "bioartificial" material (i.e. based on a natural and a synthetic polymer), both added with bioactive inorganic nanoparticles and prepared by freeze-drying and electrospinning, respectively. The two layers will be assembled by a pressure- or vacuum-assisted thermal treatment. The whole process will be developed at a pre-industrial scale.

In addition, a flexible process for the surface modification of both not absorbable and absorbable membranes will be developed, consisting of a Plasma-mediated surface "priming" treatment, followed by a

layer-by-layer (LbL) spray coating with functional polyelectrolyte layers. This coating process will allow the preparation of added-value not absorbable PTFE membranes and will also be applicable on the compact layer of bi-layered absorbable membranes. The chemical characteristics of the coating will be tailored according to the function of each membrane side: the side in contact with gingival tissue will be functionalised with natural biopolymers, such as collagen (or gelatine) and chitosan, favouring wound healing (collagen, gelatine) and exerting an antimicrobial action (chitosan). In addition, The versatility of LbL technique will allow the incorporation of nanoparticles loaded with antimicrobial drugs into the coating.

The membrane side in contact with bone tissue will be functionalised with polyelectrolytes favouring bone regeneration (such as collagen or gelatine) and inorganic fillers promoting osteointegration (hydroxyapatite nanoparticles).

Implant-based dental reconstruction is a growing market. A report on the Worldwide Dental Implant and Bone Graft Market (2nd ed., Kalorama, 2007), shows that global sales of dental implant systems are expected to reach more than $4.5billion in the next five years. Specifically, the world sales of dental bone grafts reached $130 million in 2006 and should double ($266 million) by 2012. The new technology developed by BIO-PATH project will expand the candidate pool and market opportunity for implants.

Guided tissue regeneration has become a standard procedure for periodontal regeneration therapy and GBR is getting popular because of successful implanting by using bone grafts.

The project will generate a versatile platform of advanced bioabsorbable and not-bioabsorbable MEDTECH membrane products for guided bone regeneration. The technological platform will be investigated and implemented by the manufacturing companies being the proposers of this MANUNET project, with the following product development assignment:

- Process development of bi-layered membranes at laboratory and pre-industrial scale (Laboratori Biomicron; Piedmont/Italy);
- Surface treatment of absorbable and not-absorbable membranes (Reiner Microtek; Basque Country/Spain).

The involved research and academic institutions (subcontractors) will be: (a) Politecnico di Torino (POLITO) and (b) Consorzio Proplast (PROPLAST) from Piedmont/Italy; (c) TEKNIKER from Basque Country/Spain. The well-weighted mix of industrial, research and academic organisations involved in the proposal represents a group that has at its disposal the state-of-the-art technologies as well as complementary knowledge and experience to lead the project to success.

4.4.3. Originality and innovation of the proposed approach

The project will bring innovative technologies for the development of novel functional devices with tailored and active properties for application in periodontology.

Alternatively to all existing approaches, BIO-PATH membranes for efficient guided bone regeneration will (i) exert barrier properties, avoiding the migration of gingival fibroblasts which may interfere with the bone regeneration process, (ii) promote wound stabilisation, accelerating wound healing, limiting the inflammatory reaction and exerting an antimicrobial activity, (iii) provide space maintenance, being a biological seal supporting tissue regeneration, (iv) keep mechanical stability during the regeneration process, (v) increase bone regrowth rate through a proper design of the membrane interfacial surface in contact with bone tissue.

The BIO-PATH project is aimed at the preparation of advanced bioabsorbable and non-bioabsorbable innovative barrier devices (membranes) for guided bone regeneration (Fig. 153). PTFE membranes are widely used clinically but suffer from several drawbacks, including the lack of active functionalities to promote wound healing and bone regeneration, as well as the possibility of bacterial infection due to the need for two surgical interventions. The BIO-PATH project will develop non-bioabsorbable membranes based on PTFE, surface modified by environmentally friendly techniques such as plasma treatment/graft polymerisation followed by LbL coating with functional polyelectrolytes, with the aim to improve their performance in terms of increased bone regeneration rate, antibacterial activity and wound healing rate. The proposed modifications will improve significantly the PTFE membrane functional outcome with a minimal rise in the product costs to allow their rapid spread on the market.

Bioabsorbable membrane products are available on the market such as Guidor® Matrix Barrier, Resolut® and Epi-Guide ® Bioresorbable Barrier Matrix which are layered membranes based on poly(lactic acid) and/or its copolymers. Their main drawbacks of these products are related to the lack of functionalities stimulating bone regeneration and wound healing, and exerting antimicrobial activity. The BIO-PATH absorbable products are bi-layered membranes consisting of a dense layer (based on synthetic bio-absorbable polymers or polymer blends, having tailored bio-absorption rate) fabricated by casting or melt-pressing and a porous layer prepared by freeze-drying (e.g. a nanocomposite of hydroxyapatite and gelatine/collagen with biomimetic and bioactive properties) or electrospinning (e.g. a bioartificial nanofibrous membrane added with hydroxyapatite nanoparticles).

Bi-layered membranes based on natural and synthetic polymers are highly innovative products.

A versatile process for the surface modification of either not-absorbable PTFE and absorbable membranes will be developed based on a Plasma modification/grafting, followed by a layer-by-layer spray coating of both membrane sides. The coating process will allow the modification of one or both membrane sides and the possibility to tailor the surface physicochemical properties of each membrane side by the use of different coating polyelecrolytes. For instance, the interface of the absorbable barrier devices in contact with connective tissue will be properly designed to exert antimicrobial action and to promote wound healing by a suitable surface functionalisation. Moreover, the coating process will allow the preparation of innovative PTFE membranes, able to accelerate bone repair, to promote wound healing and to exert antimicrobial action.

The expected results of BIO-PATH project are:

- The selection of a suitable biopolymer platform for the preparation of absorbable GBR bi-layered membranes;
- The set up of a pre-industrial process for the preparation of bi-layered membranes, assembling a porous and a not porous layer;
- The selection of a suitable biopolymer platform for the specific surface functionalisation of the membranes sides in contact with bone and gingival tissue;
- Product development in each stage at pre-industrial scale.

BIO-PATH strategy comprises: (i) selection of biopolymer platform beneficial for bone regeneration and wound healing process; (ii) selection of bioabsorbable synthetic polymers and their blending to modulate the mechanical and degradation properties of absorbable membranes; (iii) identification of the major microorganisms, involved in the bacterial infection at the implantation site; (iv) selection of functional materials for the coating or upgrading of the device interfaces (e.g. polyelectrolytes, such as

polysaccharides and proteins; nano-hydroxyapatite; antimicrobial agents such as Ag nanoparticles or drug-loaded nanoparticles); (v) optimization of the process parameters for the production of the (compact and porous) membrane layers and their assembly; (vi) optimization of the process parameters for the surface modification of polymer interfaces by plasma/graft polymerization followed by LbL spray coating.

A scheme on the BIOPATH membrane products is reported in Fig. 153 and Fig. 154.

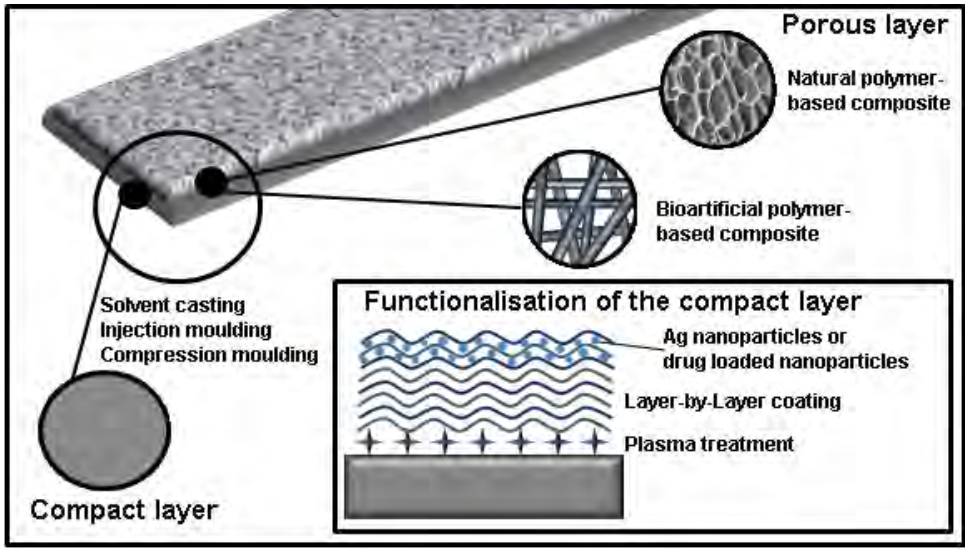


Fig. 153: Functionalised bi-layered absorbable membranes consisting of a compact and a porous layer, promoting wound healing and bone regeneration, respectively. The compact layer is functionalised by Plasma treatment followed by layer-by-layer coating.

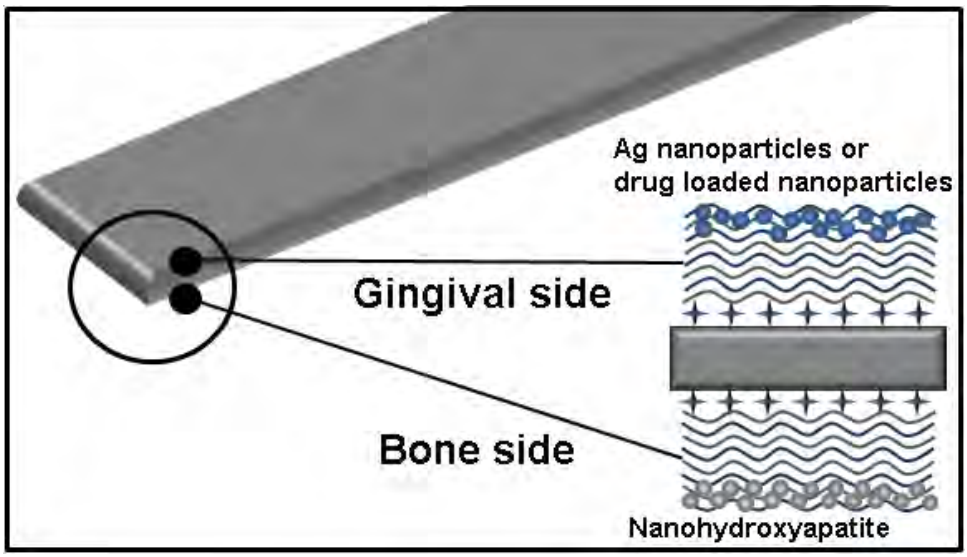


Fig. 154: Not-absorbable PTFE membranes specifically functionalised by Plasma treatment followed by layer-by-layer coating to favour wound healing at gingival side and bone regeneration at bone side.

Materials and design characteristics of the membrane devices will be selected leading to advanced MEDTECH products which are easy to be produced by manufacturing industries and to be applied by the dentists. The novel devices will be scaled up, after an accurate selection of all the necessary steps for market introduction, including: (i) definition of the industrial manufacturing steps; (ii) development of proper sterilisation and packaging modes.

4.4.4. Specific market analysis in the partners' areas

About two-thirds of Italian dental surgery centers, mainly the biggest ones, perform implantology. According to a market search, carried on by Key-Stone, an institute specialized in Health and Wellness market analysis, 24.000 dental surgery centers perform implantology, either as their own activity or using an external consultant. This value has grown by 5% per year, from 55% in 2006 to 67% in 2010. Every year, about 900000 patients need implantology treatments, with about 40 surgical interventions for each dental center. In Italy, the devices proposed in BIO-PATH project have a potential market of 900000 products per year, and are increasing by 5% per year.

Absorbable membrane market price ranges from 130 to 350 euros/unit and a similar price is related to non-absorbable ones. Considering an average price of 240 euros, the total Italian turnover amounts to 216.000.000 euros.

To estimate the size of the dental implant market in Spain, it can be considered the following approximate info (Spanish Association of Implantology source):

- In Spain every year 500,000 implants are placed (figure increased by the advance of technology and the aging of the population)
- The minimum cost of placing an implant is 1200 € (but could be much higher).

The market in Spain is cornered mainly by multinationals that import their products, but in recent years the national start-ups have developed. In this context, the objective of REINER is to compete with them in the manufacture and marketing of Dental Attachments (outside the body of the implant), with a focus on low-cost parts.

To this end, REINER has already the necessary tools and is able to produce the parts. Right now the enterprise has its own brand of products called Reiner Bio.

A critical point in BIO-PATH is the production of an improved device that can overcome the already assessed products though having a competitive price. This aim will be achieved by setting a cost-effective production process, minimizing scraps and user dependences.

Moreover, SMEs rarely have adequate R&D facilities and funding to do basic research, develop and bring to

market new products based on fully novel ideas. The MANUNET program offers a solution to this problem by providing SMEs with additional research capabilities through funding experienced and well equipped RTD performers.

4.4.5. Consortium detailed description

Consortium and project management

The management of BIOPATH project will be performed by the Project Coordinator (BIOMICRON) together with the Management Board. The Management Board will be formed by one representative of each partner led by the Project Coordinator. The Management Board of the project will cover all tasks needed for the efficient and overall coordination of the project.

Special attention will be paid on the scientific quality management with an accurate planning and control of the technical activities, whereas the exploitation and dissemination issues and financial aspects will be subjected to different regional rules.

The Management Board will provide the framework for the execution of the WP to the WP leaders and approve the results generated within the WPs and the compliance of the resource with the work plan.

Main objectives are:

- to ensure that all work meets functional requirements,
- to coordinate the work to assure flow of information between partners and to avoid duplication of efforts, to agree on press releases and joint publications and on procedures and policies for Dissemination of knowledge.

The following Management Board, including monitoring and reporting, is proposed:

Project Steering Committee

Each contractor will nominate one person who is responsible for the overall project participation of that partner. These persons will form the Project Steering Committee, which is the highest decision body within the project, including technical, controlling, planning, exploitation and financial issues.

Methods for Monitoring and Reporting Progress

At the milestone reviews the progress of the project and the outlook for exploitation of the results will be critically reviewed compared to the planning and criteria described in the work programme. Depending on the progress and the results achieved, a change in the work programme may be proposed. According to the communication strategy, all the partners will be kept fully informed about the project status, the planning and all other issues, which are important to the partners in order to obtain maximum transparency for all involved and to increase the synergy of the co-operation. The technical meetings have an important role in the information exchange. All the information, such as the minutes, reports, publications etc. will be communicated to the project co-ordinator, who will deliver the material to other partners, when appropriate. The planning of the publications to be made, presentations to be given and conferences to be attended on behalf of the Consortium will be agreed upon in the Steering Committee meetings. Between the project meetings, a frequent exchange of results will be established with the aid of Email transmission.

The main objectives of the BIOPATH management will be:

- To follow the implementation of tasks according to the detailed implementation plan, and the related level of commitments taken by the partners.
- To ensure a smooth adaptation in case of evolution of partnership needs,
- To enable flexibility in reaction to unexpected developments or new opportunities: this is facilitated by the adequacy of the partnership and the work plan structure.

The BIOPATH Consortium will implement the methodologies needed to achieve an efficient management process, and covering all the management issues: deliverables release, risks, knowledge management, Intellectual Property, decision-making and conflict resolution mechanisms. Particular attention will be paid to the efficiency of the internal and external communication.

A Quality Assurance Plan will be proposed by the partners at an early stage of BIOPATH.

This will include: (i) Time-time milestones chart; (ii) Gantt chart; (iii) reports; (iv) deliverables status. The Management part of the Quality Assurance Plan will be dedicated in particular to check the interaction between the consortium members during the work execution, check regularly the progress of the work,

detail how and when the partners must exchange the documentation, set out editorial standards for project content. This plan will enable the Coordinator to follow up and monitor the program progress. Changes that might happen in the WP output achievements will be evaluated according to their critical path, and the WP leaders reorganised in consequence. The risk management will be a major tool for performing this monitoring.

For Project Monitoring, the time-time milestones chart will be used together with an action list follow-up table; these chart and tables are monthly updated.

Program Budget Follow-Up & Monitoring will be performed, aiming at controlling the consumption of resources to achieve the targeted goals: the value of the performed work will be compared quarterly to the budget used by the WP leaders. Corrective action would then be taken in order to recover the original plan.

Scientific and technological expertise of the consortium as a whole

The Consortium is composed of two SME partners: (1) BIOMICRON from Piedmont/Italy (Project Coordinator) and (2) REINER from Basque Country/Spain. The project foresees the involvement of various research organisations: Politecnico di Torino (POLITO, from Piedmont/Italy), Consorzio Proplast (PROPLAST) and Tekniker Technological Center (TEKNIKER from Basque Country) acting as subcontractors. The project gathers an unique team of SMEs supported by skilled RTD performers with complementary skills in the field of polymer technology, biotechnology, cell biology, surface modification techniques and tissue engineering. The international/regional co-operation in the project will enable the exchange of experience and know-how in order to deliver high-added value functionalised membrane materials to the market. The business profile of the participating SMEs and the expertise of the research subcontractors covers the whole technology sequence in the production and commercialisation of membrane devices. The collaboration between industrial and research actors (industry-academia bidirectional transfer of knowledge) in Basque Country/Spain and Piedmont/Italy will contribute to the establishment of a joint technological platform for the development of novel and low environmental-impact strategies for the production of advanced absorbable and not-absorbable membranes for biomedical applications. Membrane structure will be mono-layered or bi-layered for non-absorbable and absorbable membranes, respectively. The surface functionalisation strategy will be common for both types of membrane devices.

The main motivation related with the submission of this proposal is the possibility to develop high-added value absorbable and non-absorbable membranes for GBR. The planned research activities will cover all the aspects related with the production of membrane devices.

Competences, past experiences in the field of the proposal, and role of each partner in the project

A brief profile of the SME partners and the RTD subcontractors, is provided below:

Laboratori Biomicron (BIOMICRON) - Project coordinator

Laboratori Biomicron started its activity in 1978. Its main aims are design, production and distribution of medical devices and IVD components. The main production lines involve microtiter plates having different sections of well, to perform immunological techniques, single-use elements needed in RIA kits and ELISA, medical devices for diagnostics in allergology, holders for both mechanical and biological artificial valves, kits for HPLC chromatography and BAC (blood alcohol content) (developed in co-operation with the Department of Pharmacology of San Luigi Hospital). It is involved in bioreactors applied to industrial fermentation technologies, development of related techniques and supervision of building and operation of facilities for controlled fermentation.

Reiner Microtek (REINER) - Project partner

Reiner Microtek, Polymer Microtechnologies was founded in 2008 as a joint initiative of two partners: REINER (Plastic injection and blowing) and TEKNIKER (Technological Centre) in order to develop a new Business Unit based on the Micro Injection Moulding technique and Fabrication of micro moulds and micro parts. REINER is specialized in Plastic Micro Injection Moulding with core business in designing and manufacturing of mould and tooling devices, mass production of injected micro parts and manufacturing of prototypes. Its expertise lies in Micro mould engineering, Micro part engineering, Micro Injection and the plastic sector in general. REINER already developed products like Orthodontic Brackets, Drug Microdosifiers, and parts of around 10 mm3 and precision of 1 micron. One of the main advantages of REINER is their experience in the product development from the beginning to the end. Main REINER expertise involves Micro Moulding and Micro Injection processes and equipment and the Plastic sector.

POLITECNICO DI TORINO (POLITO) – BIOMICRON Subcontractor

The Industrial Bioengineering Group has a long and proud history at the Politecnico di Torino where the activity of the Group started in 1978 and was since devoted to both education and research. Today, the Group's research issues are related to engineering in surgery; computational biomechanics; prostheses; multiscale and multiphysics modelling; biomolecular, cellular and tissue engineering; bioartificial systems constituted by the synergy of biological and artificial components as in cell on a chip systems and in bioartificial materials; bioinductive interfaces; diagnostics and detection systems; technologies for tagging, handling and moving at nanometric scale; molecular motors and so on. The group has been actively participating in research programmes in the 6FP (The Streps Hipermax, Photonanotech), the COST actions 847 and 868 (in the latter as vice-coordinator), collaborations with medical industries on regional and national projects and two Manunet Projects (NANOWELL and BIODRESS).

Consorzio Proplast (PROPLAST) – BIOMICRON Subcontractor

PROPLAST is a no-profit R&D and consultancy body founded and sustained by about 180 enterprises all operating in the plastic sector. Proplast offers the following services: product design, mould design, mould flow analysis, mould manufacturing, laboratory testing, injection moulding optimisation, compounding of materials. PROPLAST is the most equipped engineering body in the fields of plastics in Italy, with 3500sqm facility and 40 people operating as technicians, engineer and chemists. In the mould making sector, PROPLAST is well equipped with all equipment that are normally used in mould making process (CNC milling and grinding, Spark erosion, etc). Mould flow and several simulation software are also available.

PROPLAST is i) the national reference point for experimental and research activities on polymer processing, ii) promotes co-operation between national and international companies, athenaeums and other business, formative and research entities, iii) offer services to all sizes of companies, with the aim to contribute actively to the economic development, stimulating and facilitating the processes of innovation, technological development, technology transfer, enterprise creation. PROPLAST has been nominated by the Piedmont Region as a Innovation pole for advanced plastic materials and related technologies. PROPLAST manages a network of members with 180 enterprises, 7 industrial associations, 13 universities and has – therefore – a relevant exploitation potential.

TEKNIKER - REINER Subcontractor

TEKNIKER is a Mechatronics, Manufacturing Technologies and Microtechnologies centre in manufacturing technologies, i.e. all areas concerned with products, processes, production machinery, handling and all-round management of product life-cycle and its optimization. The μ-Technologies Department has the finest set of ultra-precision and μ-manufacturing equipment in Spain and it is staffed by people highly trained at the most renowned European centres of excellence. In commonly adopted terminology, TEKNIKER is a manufacturing or industrial production and design centre. It mainly specialises in: (i) Designing consumer and industrial products; (ii) Solving problems relating to friction, wear and lubrication; (iii) Incorporating information technologies and communications in the plant; (iv) High precision, miniaturisation and micro/nanotechnologies. TEKNIKER has experience in surface modification at the nanoscale: one of the traditional activities in this area consists of the vapour phase deposition technologies, especially Physical Vapour Deposition (PVD) and sol-gel technology to synthesise multifunctional coatings. In addition, TEKNIKER has competence in the mechanical (adherence of coatings, surface hardness, elastic properties, etc.) and physicochemical characterisation (GDOES analysis, microscopic observation, SEM, etc.) of modified surfaces. TEKNIKER belongs to alliance IK4, a project that aims to contribute to strengthening the innovative capacity and competitiveness of enterprise.

Main project activities and partners responsibilities in the proposal

Main role of BIOMICRON is the development of a process for the preparation of bi-layered membranes by exploiting the subcontractors' experience and in collaboration with REINER and TEKNIKER. In detail, BIOMICRON, REINER, TEKNIKER, POLITO and PROPLAST will select synthetic polymers (e.g. polylactide, polycaprolactone) and prepare polymer blends. Polymers and blends will be characterised for their mechanical and physicochemical properties. Compact membranes will be prepared by film casting (POLITO), compression-moulding (PROPLAST) and injection moulding (REINER). Dense membranes will be characterised for their mechanical and physicochemical properties. Natural polymers (proteins and polysaccharides) will be selected by POLITO and BIOMICRON. Porous membranes will be prepared by POLITO and BIOMICRON (in collaboration with REINER) by freeze-drying of natural polymers (even in the form of blends) and electrospinning of natural and synthetic polymers (even in the form of bioartificial materials). Natural polymers and bioartificial materials will be added with bioactive inorganic particles, such as nano-hydroxyapatite. The porous membranes will be characterised for their mechanical and physicochemical properties. The process for the assembly of the two membrane layers will be optimised by PROPLAST (in collaboration with REINER and TEKNIKER) based on a vacuum- or pressure-assisted thermal treatment. The biocompatibility of the single membrane layers and the bi-layered membranes will be evaluated (BIOMICRON). BIOMICRON will deal with process design at laboratory and pre-industrial scale in collaboration with REINER. In addition, as a coordinator, BIOMICRON will be responsible of project management.

Main role of REINER is the development of a versatile process for the surface modification of polymer membranes, exploiting TEKNIKER experience in the field of surface modification of polymer substrates at the nanoscale. In detail, biomolecules for surface modification of the membranes (mainly polyelectrolytes) will be selected with the help of POLITO, which has a recognised experience in the surface functionalisation of biomedical devices. A process for the surface modification of membranes, based on Plasma treatment and layer-by-layer spray coating, will be optimised by REINER, BIOMICRON, TEKNIKER and POLITO. The process will be applied to both absorbable (bi-layered membranes developed in the project) and not absorbable (commercial PTFE) membranes. In the case of the bi-layered membranes developed in the project, only the exposed dense membrane surface will be modified to favour wound repair. Antimicrobial agents for the functionalisation will be selected by POLITO and BIOMICRON (in collaboration with REINER).

The modified membranes will be characterised for their physicochemical and mechanical properties as well as coating stability. The ability of the coatings to support keratinocytes and fibroblasts adhesion and proliferation on the membrane side in contact with gingival tissue and osteoblasts attachment and proliferation on the membrane side in contact with bone tissue will be evaluated by BIOMICRON. REINER will deal with process design at laboratory and pre-industrial scale in collaboration with BIOMICRON. REINER will also collaborate in the project management and will coordinate Dissemination and Exploitation items of the project.

Project management capacity of the coordinator

The project consortium recognises the importance of good management skills in ensuring that the research programme proposed is completed in a timely, professional manner, with a clear structure for distributing the work activities logically amongst the consortium partners, monitoring progress and production of deliverables. The partners have significant European management experience, which will be utilised to the full to ensure that the project is completed successfully, with deliverables produced on time and within the budget. Effective project control and communication within the project is achieved through a well-organised management structure. This structure will ensure that there are no problems of communication between the collaborating partners within the Consortium, and that work is reviewed and feedback obtained on a regular basis. It has to be noted that many of the partners have already collaborated in several projects (both research projects and commercial or industrial projects), so a good and fluent communication of the consortium is assured. BIOPATH will be managed by BIOMICRON in the person of Giuseppe Falvo D'Urso Labate, who has daily contact with partners from different running R&D projects. This permits a continuous upgrading of the management skills and capabilities to interface with different companies from different countries and involved in various business, but all focused in a common target. In the BIOPATH project, BIOMICRON will follow the newest and most advanced model for excellence and quality of management, based on management by processes and facts. Thus a continuous process will monitor and improve the management of the project, on the basis of indicators that measure the performance of the results produced.

Consortium Agreement

The cooperation terms will be regulated in the Collaboration Agreement (CA), that will be presented in the form of definitive draft with the full proposal. The collaboration terms establish the royalty- free transfer of pre-existing know-how necessary to carry out all the activities proposed, which shall be utilised under goodfaith principles. Moreover, the CA establishes the terms for using and disseminating the knowledge generated during the project. In general terms, all partners will be free for using and disseminating all generated knowledge, unless otherwise agreed (e.g. commercially-sensitive know-how).

Concerning Intellectual Property Provisions, the Collaboration Agreement presents particular emphasis on exploitation interests, licensing policy and Intellectual Property Rights (IPR) protection according to the interest of the Consortium partners. The consortium will assure that innovations and patentable results developed in the project are protected in international law, and that such protection will ensure that the maximum possible benefit remains with the consortium partners and European industry. The IPR share between the partners, which has been discussed, will be frozen at the agreement Finalisation time. Regarding the concepts, models, technologies and process obtained as results of the studies undertaken during the project, they are expected to be property of Consortium Partners that developed them. Access right to the other partners can be released, if requested, because there is not concurrency between participants. The objective of this part is to set the basis of a policy for exploiting the results of the BIOPATH project respecting the rules of the MANUNET Contract.

Partners must have a clear view of the essential pre-existing know-how (PEK) that is brought to the project, the expected outcomes, and benefits for their organizations. This means that the success of BIOPATH cannot be evaluated just by counting the number of patents generated. The preparation of a register of PEK has already started; all partners have been invited by the Project Coordinator during the preparation phase to send a registered letter declaring their PEK and specifying whether they intend to exclude any part of it from the obligation to grant access rights to the rest of the consortium. All these letters will be included in a register that will be distributed among the partners and then included in an Annex to the Consortium Agreement (CA). The CA contains a clause which regulates the update of the PEK register in the event that a new partner joints the consortium. Intellectual outputs from the project will include formally protected knowledge, tacit knowledge, commercial knowledge of markets and consumers and other non-scientific and technological knowledge, and will contribute to the pool of public knowledge. The IPR Management strategy will take the above-mentioned issues into account and will be integrated with the CA. This is the legally binding document where rights and responsibilities are clearly defined and agreed for the life of BIOPATH. The CA forces the participants to identify their own interests, rights and responsibilities at the outset and to recognize those of others within the project, codifying these within a legally binding document. Collaborative structures and legal agreements are decided in order to ensure the rights of members to use outputs and the obligation to provide access to necessary background.

The Consortium has an IPR Manager for all IPR related tasks and questions. If, in the course of carrying out the joint work, an invention or design is made, and if the features of such a joint invention or design are such that it is not possible to separate them for the purpose of applying for, obtaining and/or maintaining the relevant patent protection or any other IPR, the parties concerned agree that they may jointly apply to obtain and/or maintain the relevant right together with any other parties concerned:

The parties shall seek to agree between themselves arrangements for applying for, obtaining and or maintaining such right on a case-by-case basis.

4.4.6. Expected results and exploitation

In the last years, the EU manufacturing sector has been suffering high financial losses due to the competition from low-wage cost countries, like those from South East Asia. Therefore, the EU SMEs need to address strategic challenges to survive in the global market.

The proposed BIO-PATH project will have a high market impact for the SMEs participating in the project, as it will allow the implementation of several strategies that are a key to competitive success:

- Product differentiation, through the introduction of technical features meeting specific market needs;
- Cost minimisation through the development of cost-effective products by novel manufacturing approaches, in light to the added-value imparted to the new MEDTECH products;
- Involvement, utilisation and training of the labor force to maximise competitiveness.

The collaboration among BIOMICRON and REINER SMEs with academic and research staff from TEKNIKER, PROPLAST and POLITO will allow the technicians of the SMEs to acquire new knowledge and expertise and will contribute to motivate them in their activities, encouraging further research and development activities.

A Collaboration Agreement will be signed before the project starts by all involved organisations (i.e., by both the project partners and the subcontractors), in order to cover all exploitation issues of the project, such as patent applications, licenses, etc.

After potential patent applications have been filed, the results obtained in the project will be disseminated through several routes, including scientific and technical papers and presentations at conferences and workshops organized in the EU Member States. The transregional and transnational character of the industrial, academic and research organizations involved in the project will be beneficial to the spreading of the project outcomes to different geographical areas, addressing both research and industrial actors. The dissemination of the project will encourage other industries that are external to the consortium to realize new research to get the leadership in the field.

From a social point of view, the improvement of medical devices may reduce sanitary costs, patients' discomfort and pain.

The novel products developed in the BIO-PATH project will be:

- Innovative bi-layered absorbable membranes with proper mechanical, morphological and physicochemical characteristics for GBR (BIOMICRON);
- Development of a versatile processes for GBR membrane surface functionalisation to improve device functional performance (REINER).

The production processes developed will be cost-saving in the whole production chain, and cheap materials will be selected when possible, in order to assess competitive industrialization.

The project coordinator BIOMICRON has already identified two main distribution channels for the product: the first one, related to Italian market (mainly North-East of Italy), is expected to absorb approximately 900 products/year, the other one, related to French market, will absorb 1.200 products/year. So, about 2.100 products are supposed to be produced and placed on the market during the first year after product development; this value is expected to increase by 5% for each subsequent year. Furthermore, market strategies are in progress in order to open other Italian distribution channels and to increase the number of foreign customers.

The new production line will need new competences within both SMEs, thus increasing the number of employees in both BIOMICRON and REINER.

Current project results will make REINER not only increase its competitiveness, but also its ability, thanks to the launch of new products with higher added value through more efficient processes, and under greater control. A positive answer from market is expected in a short period, strengthening REINER presence in biomedical sector. This sector is the main application field for micro-parts at European level. The opportunities that this market offers are its growth potential by offering a variety of applications and profitability, as the price is a secondary aspect.

This project will provide a strong impetus in the development of self-knowledge in the face of these sectors. Specifically, the experience obtained on different biocompatible polymers can be key to the future development of the company. It could expect REINER gains an increase of its market share by 20% approx. which translates into a significant financial growth for the company

In short, REINER will diversify its portfolio, obtaining results in a diversified portfolio of orders, generating more activity and work (internally). Forward looking, this diversification and flexibility of products and processes will allow the entry of potential customers from biomedical sector (for example guided bone regeneration, where the applications of biocompatible polymers, is taking relevance).

4.4.7. Project statistics

The detailed description of work and the GANTT diagram are not reported here. Nevertheless, the BIO-PATH project involves 78 person months; Table 25 and Table 26 show the total cost per partner per year and the analytical cost plan respectively. 40% of the project will be financed by European Community.

Table 25: Total project cost per partner per year.

	Costs Year 1 (Months 1-12)	**Costs Year 2** (Months 13-24)	**Total Costs**	**Requested Funding**
Partners role	(Euro)	(Euro)	(Euro)	(Euro)
Coordinator BIOMICRON	197.595	197.595	395.190	158.076
Partner 2 REINER	90.000	90.000	180.000	90.000
Total	**287.595**	**287.595**	**575.190**	**248.076**

Table 26: Analytical cost plan.

	Personnel	**Overheads**	**Travels & Subsistance**	**Material & Supply**	**Equipment**	**Subcontractors**	
Partners role	(Euro)	(Euro)	(Euro)	(Euro)	(Euro)	(Euro)	(Euro)
Coordinator, BIOMICRON	200.114	37.000	10.000	45.000	28.000	75.076	395.190
Partner 2 **REINER**	96.000	24.000	10.000	20.000		30.000	180.000
Total	**296.114**	**61.000**	**20.000**	**65.000**	**28.000**	**105.076**	**575.190**

5. Development of tissue engineering bioreactors

Provided the studies reported in Chapter 2 and the context analysis in Chapter 3, the development of bioreactors for tissue engineering are considered feasible for a SME.

Two sector were chosen, the cardiovascular sector, because of the large size of its potential market and the small amount of competitors in this field, and the orthopaedic sector, because bioreactors for orthopaedic tissue engineering are already present on the market, that showing an easier commercialization of this product. Moreover, sensors and parameter control are always kept as a main feature, aiming at making bioreactors user-independent, in order to achieve automation, this being one of the limits that hamper bioreactor diffusion, as stated in paragraph 3.3.

5.1.State of the art of bioreactors for cardiovascular tissue engineering

The heart is one of the organs that has the least ability to regenerate. For this reason, any damage (due to ischemia, viral infection or other diseases) which causes a significant loss of cardiac cells, leads to "overloading" of the healthy areas of the myocardium, causing an evolution towards an irreversible collapse of the cardiac muscle.

Due to the (i) remarkable rapidity of loss of functioning caused by cardiovascular diseases associated with congestive heart failure, (ii) shortage of donor hearts for transplantations, (iii) complications and long-term collapse of transplanted organs resulting from immunosuppression, it has become increasingly compelling to research a new treatment paradigm based upon the convergence of the most recent findings from basic research and technology in the field of life sciences.

It is in this context that a new biomedicine discipline has emerged and developed - regenerative medicine - stimulated by the recent huge discoveries in stem cell biology, cell therapy and tissue engineering. One of the primary objectives of regenerative medicine in the cardiovascular field is the development of a cell replacement strategy that encourages the repopulation of damaged tissue with new contractile cells, to restore the physiological functioning of the heart. The "repair" of the damaged heart tissue is, therefore, pursued by transplanting engineered functional heart tissue, i.e. a tissue obtained in vitro and made up of healthy, functional and proliferating cells that are capable of restoring the vitality and functionality of the native tissue.

In recent years, significant investments in research have been made worldwide to develop cardiac constructs to be implanted into ischemic hearts. The literature shows that there are cardiac constructs with distinct structural and functional properties, depending upon the culture conditions adopted (type of substrate/scaffold, culture medium, static or dynamic culture and perfusion).

The main problems found in attempts at engineering heart tissue stem from the intrinsic complexity of the latter. In fact, in addition to its characteristic contractile activity, normal functioning heart tissue must contain the correct proportion of stroma (connective), with supportive function, and blood vessels which bring oxygen and essential nutrients. Heart cells must also establish appropriate electric junctions so that the contractile impulse is spread evenly and there are no problems of arrhythmias which may aggravate the disease, giving rise to fatal events.

In order to obtain correct constructs, advanced engineering tools have been developed (bioreactors), capable of creating a dynamic culture (i) which ensures the correct transport of nutrients (in particular, oxygen) and metabolites to and from the cells (298), (299), (300), (301) and (ii) which applies a dynamic, mechanical stimulus (61), (237), and/or an electrical stimulation (192), (237), in order to provide appropriate physiological stimuli to the cells in culture.

These culture models, developed in order to stimulate the heart constructs mechanically during their maturation, have allowed us to demonstrate that dynamic conditioning, with relative application of well-known and reproducible load histories, facilitates the production of myocardial tissue that is more organised and, as a consequence, functionally more efficient. In particular, the application of cyclical mechanical stretch regimen to human cardiac cells (302) or those of rats (303), (304) has allowed for the generation of three-dimensional cardiac constructs, improving the proliferation and structural organisation of the cells and inducing the formation of an organised extracellular matrix. In addition, it has been demonstrated that electrical stimuli, applied to cardiac cells in culture, encourages the functional optimisation of the resulting tissue, inducing cellular alignment and coupling, increasing the amplitude of the synchronous contractions and improving the level of structural organisation of the sarcomeric apparatus (192). The electrical stimulus has also been used to synchronise the contraction of cells in the engineered cardiac construct (305).

Finally, it has been demonstrated that the use of a direct perfusion during culture encourages uniform distribution of the cells across the whole thickness of the construct, guaranteeing the retention for long periods of three-dimensional cell cultures and preventing conditions of hypoxia and necrosis (306).

The context described above clearly identifies the complexity that regenerative cardiovascular medicine must address in order to create cell cultures in an appropriate in vitro environment, which is able to encourage cell adhesion, proliferation and differentiation, in addition to synthesis of growth factors for the development and maturation of the tissue itself and the subsequent integration of engineered tissue into the recipient myocardium. Therefore, it is clear how much the success of regenerative cardiovascular treatment is closely related to an appropriate selection not only of the cells and substrate, but also the type of dynamic conditioning so as to maintain the cells in conditions which maximise their capacity to perform their physiological role, ensuring their adequate in vitro maturation prior to implantation.

This level of complexity requires a method that combines the experimental results with intelligent systems in order to systematically determine (i) maintenance of the physiological conditions in the cell/tissue environment, (ii) efficient transport of nutrients, oxygen, metabolites and growth factors among the cells and their environment, (iii) the presence of fundamental regulatory factors in cardiac development. All this must be accompanied by a control system able to regulate, in a repeatable, automated manner, adequate mechanical and/or electrical stimuli.

While scientific research in this field has already produced various results, from a business point of view, the bioreactor product is still in the most elementary stages.

Globally, the commercial bioreactor market is dominated, with the exception of some cases such as the BOSE-Electroforce© (Fig. 155a), by SMEs predominantly based in the United States and, to a lesser extent, in Europe. The latter include the Synthecon© (Fig. 155b), the New Brunswick© (Fig. 155c) and the TGT© (the latter derived from the afore-mentioned United States' BOSE). As regards Italy, on the other hand, industrial manufacturers of bioreactors for tissue engineering are virtually absent or, at most, are present in embryonic stages in the form of university spin-offs or industrial start-ups, none having yet acquired the distinction as established manufacturers.

Fig. 155: a) BOSE-Electroforce multi-chamber bioreactor; b) Synthecon rotating bioreactor; c) New Brunswick bioreactor

Notwithstanding the fact that the prospects opened up by tissue engineering have aroused much enthusiasm and generated significant investments, the products linked to it currently still come up against the economic evidence of an uncertain costs/benefits ratio and the incapacity to amortize, in the short-term, the profuse investments, also due to a relatively lengthy time-to-market. The limited commercial progress achieved to date is also attributable to the fact that the procedures for generating engineered tissue are generally based upon procedures that are almost completely non-automated which, with a view to large-scale production, constitute an excessive burden in terms of costs and present intrinsic risks of contamination and lack of uniformity.

Conversely, a closed production system, standardised and independent of the operator, based upon automated production elements, like that of bioreactors, has huge benefits in terms of cost, safety and regulatory compliance. Notwithstanding the high costs of development, this approach has great potential for improving the costs/benefits ratio of the manufacturing process, able to maximise also the benefits deriving from a relative economy of scale.

5.2. Fund raising strategy

The fund raising strategy was based on three opportunities, one given by Italian Ministry of Economic Development, one by Regione Sardegna, and the third by Regione Piemonte. So, three development projects where tailored to those Calls:

- PROBING (Prototipo di Bioreattore Ingegnerizzato), in the ambit of “ACCORDO-QUADRO MI.S.E.-ICE-CRUI Attuazione 2010” Italian Ministry of Economic Development, that aims at developing a bioreactor for tissue engineering of cardiac muscle;
- Pro.Va.De.Ri.Va.Sa (Vascular prostheses through Innovative Decellularization and Repopulation of Blood Vessels), in the ambit of “Polaris Research Incentive 2011 – Sardegna Research – Regione Sardegna”, that aims at developing a bioreactor for tissue engineering of blood vessels;
- BIOBONE (Bioreactor for biased osteointegration) , in the ambit of “Programma Operativo Regionale “Competitività regionale e occupazione” F.E.S.R. 2007/2013 – Regione Piemonte”, that aims at developing a bioreactor for tissue engineering of bone.

Being fundamental their technical feasibility and economic affordability, these projects have been written keeping in mind the importance of IPR protection and the economic impact they bring.

They are described in the following paragraphs.

5.3. PROBING

5.3.1. Project partners' list

1. Politecnico di Torino (POLITO)
2. Università di Torino – Centre for Molecular Biotechnologies (BIOTEC)
3. Imperial College London (ICL)
4. Laboratori Biomicron s.r.l. (BIOMIC)

5.3.2. Project detailed background

In order for the bioreactors to become widespread, it is essential to introduce bioreactors which are simpler than those currently available on the market and are user-friendly even in the research phase, since the continuous use by operators of complicated instruments discourages their large-scale introduction.

The lack of a sufficient number of costs/benefits analyses in the context of clinical effectiveness, together with the scarcity of a relevant statistical sample, also hinders the attempt at finding a plausible determination of the probability of remunerative cover for the activities of production and surgical implantation of engineered products, something which strongly affects the credibility of commercial plans based upon the widespread use of tissue engineering. Given the problematic issues in this economic model and the highly discontinuous market demand for bioreactors, the hesitancy of the industry in undertaking the marketing of the latter is quite understandable, as is the limited investment of significant resources to develop production technologies in this field.

A strategy which involves closer interaction between scientists and industrial partners would certainly facilitate the design and development of bioreactors that are easier to use and less expensive than models currently available on the market, such as those produced by small and medium industries and, to a much lesser extent, by large industries (which are characterised by a high degree of complexity). The simplification process involves identifying the cell processes, the culture parameters and the optimal construct parameters which must be monitored and maintained in order to standardise production and provide reliable data on quality and traceability, minimising at the same time the risks, costs and complexity of use.

The development of bioreactors that are simpler to use presents for the industry the further benefit of being able to implement production strategies which require a lesser number of operators, facilitating the design, limiting the number of technical specifications and thereby significantly reducing production and operational costs.

In conclusion, the cardiovascular bioreactors market is predominantly untouched and constitutes a very interesting niche in which to be positioned, by taking advantage of the ascent of the tissue engineering market and the limited problems related to lack of competition.

5.3.3. Project objective

The PROBING project aims at developing and optimising a prototype bioreactor for regenerative cardiovascular medicine through technological transfer from the universities to the company, in a highly innovative sector in rapid expansion on the global market.

To this end, a scientific/industrial multidisciplinary cluster will be established, based upon collaboration between two Italian universities (Politecnico di Torino, Università degli Studi di Torino), a foreign university (Imperial College London) and an Italian SME (Laboratori Biomicron srl). This cluster is aimed at developing

a virtuous cycle of shared research and innovation, with a view to technological industrial development and internationalisation.

Thanks to the careful selection of partners and the thorough planning of their interaction, the project aims at responding to long-term requirements such as generating new knowledge, innovation in scientific and industrial research and a competitive return for the company by developing an innovative prototype with highly technological content in the field of biomedical technology for cardiovascular treatment. PROBING, in fact, will involve activities of creating, optimising and validating the prototype; activities which require, for their implementation, broadly multidisciplinary skills. A fundamental factor will therefore be the transfer of knowledge and technological competences from the academic world to the business sector, with the further opportunity for the company, thanks to the consolidated relationships of foreign collaboration initiated by the university, to broaden the potential for implementation abroad by creating an innovative product that can be patented, with consequent competitive penetration and growth in the international markets.

The result of the PROBING activities will provide an immediate impact in terms of:

- research product, which increases, on one side, the capacity for the company to innovate and, on the other, the level of knowledge of the universities involved;
- synergy between the universities and the company;
- potential for internationalisation for the company involved.

Over time, this immediate impact will be supplemented by the effects of developing the prototype, which in future will be able to enter the market to the benefit not only of the company but also public facilities such as the health systems. The PROBING project will therefore be held as an example of how it is appropriate and effective to lead industries and research facilities present in Italy to a level of successful competitiveness and excellence in an international context.

Another positive impact of the project stems from the involvement of two Italian universities and a foreign university, whose ability to provide higher education will ensure a transfer of skills between academia and industry in a highly interdisciplinary and multidisciplinary collaborative context. This aspect is afforded great attention in the project, which is intended, alongside the development of the prototype and the possible filing of patents which will ensure competitiveness and business to the company involved, to fully achieve objectives with a broader scope, such as the growth and valorisation of human capital and the establishment of effective fields of communication and collaboration between the various disciplines and between academic and industry research.

With regard to the scientific and technological aspects, which will be described in detail below, the project is positioned within the field of regenerative cardiovascular medicine which aims to “repair” damaged heart tissue by transplanting functional engineered heart tissue, obtained in vitro and made up of healthy, functional and proliferating cells able to restore the vitality and functionality of native tissue. In this context, PROBING aims to develop a prototype of an advanced dynamic cell culture tool (bioreactor), which is essential for the formation and in vitro growth of engineered heart tissue.

The complexity of the tissue in question raises a series of technical and technological critical issues which will be addressed by a new methodological approach based upon integrating the following actions: (i) “intelligent” planning of experimental activities for systematic exploration of all possible design variants, conducted by developing and adopting advanced decision support tools which perform the lead role; (ii)

development and optimisation of technologically advanced tools in order to develop a bioreactor prototype capable of producing engineered heart tissue which has a high probability of successful implantation into the patient, as well as subsequent integration and functioning in the recipient myocardium.

The dynamic bioreactor for developing cardiovascular constructs will be made up of two main units: a unit dedicated to the dynamic culture of the tissue (culture unit), and another dedicated to monitoring and controlling the culture itself (control unit). The task of the control unit will be to record and process data measured inside the culture unit, within which new generation sensors will be installed to measure the parameters able to monitor the metabolic and/or functional characteristics of the culture constructs (Fig. 156).

The design specifications for the new bioreactor will be defined based upon a bottom-up approach, commencing from an analysis of the needs of the final user and focusing attention primarily on the culture chamber, which must guarantee the necessary requirements for the effective development of cardiovascular cultures. All the design choices will be validated each time, so as to identify and resolve problems and technological limitations, leading to a final version of the prototype bioreactor capable of delivering the required performance.

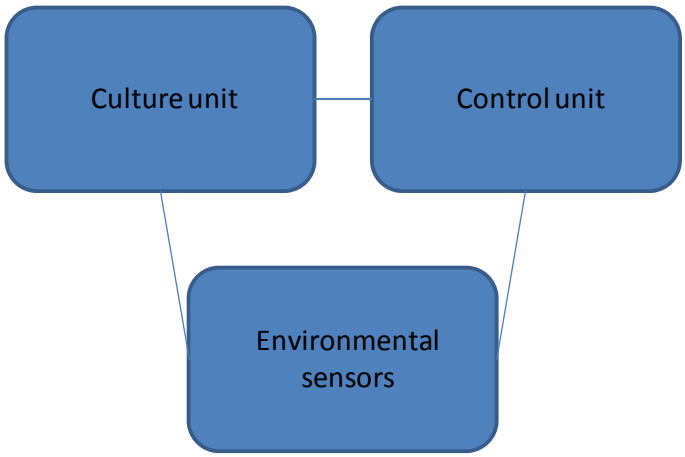


Fig. 156: Basic flow chart of the closed control of a bioreactor.

As regards the measurements of a physical nature (force/deformation), it will be necessary to define the test and calibration procedures which will ensure the reliability of the data originating from the culture chamber. In fact, in applying commercial force sensors to the bioreactor in the development phase, such sensors not capable of being placed directly inside the culture chamber due to the hostile environment and hermeticity of the latter, measurement errors occur which are connected to the presence of a certain number of mechanical interfaces between the sensor itself and the culture sample. Therefore, tests will need to be conducted to establish the necessary corrective factors to be applied via software to reduce the measurement error. Similar problems will also be addressed for other types of sensors.

With regard to chemical sensors (pH, oxygen and carbon dioxide concentration), all invasive probes used must guarantee sterility and resist sterilisation techniques. The sensors must also provide for the direct measurement of the variable without the use of additional reagents, with a high resolution that can detect the slightest changes in the variables in question.

In terms of monitoring parameters such as partial oxygen and carbon dioxide pressure, micro-fluidic technologies will be used, since these types of sensors, which can be positioned outside the incubation

chamber, have the advantage of simplifying the project from the point of view of sterility requirements. The identification, during the course of the project, of the most suitable sensors for culture monitoring purposes will be reflected in the identification of the specific techniques of the feedback system, which must continually adapt the stimulation parameters to evolution of the culture.

5.3.4. Consortium detailed description

Politecnico di Torino – Department of Mechanics (POLITO)

It brings to the project:

- engineering skills in computational modelling (structural, fluid dynamics, multi-physics);
- skills in developing advanced dynamic culture systems (bioreactors), based upon more than a decade spent developing mechanical systems for cardiovascular in vitro simulation.

It contributes to the project by:

- acting as project leader and co-ordinator;
- identifying the technical specifications and developing the prototype;
- developing the perfusion system;
- selecting appropriate sensors or adapting commercial ones;
- developing custom control software;
- carrying out the fluid dynamics study of the cell/scaffold construct inside the bioreactor;
- modifying the prototype based upon feedback from the experimental cell tests.

Università di Torino – Centre for Molecular Biotechnologies (BIOTEC).

It brings to the project:

- skills in the culture and in vitro differentiation of stem cells;
- skills in analysing the intracellular signalling pathways which determine the cell proliferation and differentiation;
- skills in animal models of heart failure;
- skills in analysing heart functioning in mice.

It contributes to the project by:

- conducting tests on cell growth, differentiation and survival using the prototype produced;
- carrying out histological analyses of the three-dimensional organisation of cultivated cells;
- providing feedback on the ease of use and versatility of the prototype.

Imperial College London (ICL)

It brings to the project:

- skills in fluid dynamic computational modelling.

It contributes to the project by

- participating in the computational fluid dynamic study of the cell/scaffold construct inside the bioreactor.

Laboratori Biomicron (BIOMIC)

It takes part in the project by providing:

- consumption materials and semi-finished products;
- electronic material and sensors;
- anything that proves necessary for developing the prototype bioreactor.

5.3.5. Expected results

The Project aims at producing a bioreactor prototype, by combining the research experience of the Politecnico di Torino, Imperial College London and the Centre for Molecular Biotechnologies of the Università di Torino with the business skills of the company Laboratori Biomicron srl, in order to facilitate the competitive growth of the latter on an international market with high technological content and in strong expansion, such as that of regenerative medicine.

The significance of this project in the scientific and technological field lies in the application of a systematic and multidisciplinary approach, which innovatively integrates engineering and biological expertise to create a system of cardiac tissue culture which is highly innovative compared to those present on the market.

The expected results and innovations are:

1) Progress in designing and developing bioreactors
2) Innovation for pre-competitive product development
3) Filing of trademarks and patents
4) Technological transfer between the Universities and the Company
5) Commercial implications
6) Designing an intelligent system to simulate, integrate and guide experimental activities in engineering cardiovascular tissues
7) Progress in identifying the optimal conditions for dynamic cell cultures

Progress in designing and developing bioreactors

The project aims to develop a prototype of a dynamic bioreactor, equipped with optimal solutions as regards both dynamic tissue culture and the sensors fitted on the bioreactor to develop a system of feedback control for cell growth.

The expected scientific and technological implications are the broadening of knowledge in relation to the dynamic culture of cardiac muscular tissue (choice of culture conditions and electro-mechanical stimulation parameters), new solutions for bio-sensors and bioreactor actuation and an innovative control of tissue differentiation and growth.

The project will increase knowledge in design and development techniques both in relation to hardware (HW) and software (SW). For both aspects, the expected results are listed below.

- HW, dedicated to dynamic tissue culture, must guarantee:
  - hermeticity and seal of the organs and, in particular, the culture chamber;
  - sterilisation by way of autoclave of all components of the culture unit;
  - compatibility with the cellular environment of all invasive components in the culture chamber (in particular, the sensors)
  - maintenance of sterility inside the culture chamber during operation;

- stability of the control logic and functioning of the mechanical actuator (the mechanical system must guarantee seamless operation in nominal conditions of mechanical load for 30 days).
- compliance with the waveforms imposed and testing of the stimulation control logic (control subsystem-electrical side).

SW, dedicated to dynamic tissue culture, must guarantee consistency and correct functioning of the feedback loop which integrates the data from the in vitro experiments and the in silico computational models, thereby leading to an automatic cell culture process.

Innovation for pre-competitive product development

An integrated HW+SW prototype device will be produced which is able to realise the development of culture techniques. This device on one side will consist of a pre-competitive product already ready for marketing; on the other, it may be used as a model to construct further bioreactors for engineering other tissue categories.

Filing of trademarks and patents

The study will lead to the presentation of at least one trademark connected to the product and to the identification of patents which the company involved will use in its subsequent distribution.

Technological transfer between the Universities and the Company

The sector of regenerative medicine and, even more so, the manufacturing of bioreactors is at a stage where integration between the university system and the business world is an essential condition for developing a product that can be industrialised. In this sense, the transfer of knowledge between the academic world and industry, just like the creation of joint working groups of an international nature becomes a key to the success of projects in sectors with high technological content. In terms of bioreactors, such integration becomes even more necessary in view of the fact that the university system in a national and international context is the primary recipient of the product itself.

Commercial Implications

The project will support an Italian company in positioning itself in the European bioreactors market.

The company has the benefit of not having to invest in the costs of personnel in research and development in a field in which economic analysis is lacking in reliable data. The project gives the company the chance to interact directly with the most advanced research laboratories, developing in the fastest and most effective way possible an instrument whose distribution is planned within the coming 3-5 years.

Designing an intelligent system to simulate, integrate and guide experimental activities in engineering cardiovascular tissues

This project aims at developing a computational environment based upon the integration of information originating from the bioreactor and the cell population. The computational models will allow for the simulation of interactions between the cells and the culture environment and will be able to predict overall behaviour emerging in the cell culture itself. During the course of the project, the models will be refined based upon the laboratory experiments which will allow for both the development and the validation of the models themselves.

The strong synergies developed between the partners will help to create a continuous exchange of information between the in silico model and the in vitro/vivo experiments.

In order for the objective to be achieved, the computational model must:

- Provide new relational elements between cellular interaction and tissue development.
- Allow for the creation of an *in silico* laboratory, which will guide or preliminarily replace the in vitro experiments, improving efficiency by saving costs and time.
- Be inserted into the bioreactor control software allowing for continuous adjustment of the culture parameters to obtain a cell construct which reproduces the characteristics of cardiac tissue.

Progress in identifying the optimal conditions for dynamic cell cultures

From the point of view of cell cultures, the expected results of the project are:

- identification and selection of appropriate cell mixtures made up of cardiomyocytes and stem cells suitable for generating, once seeded on the scaffold and cultivated inside the bioreactor, a complex and vascularised tissue able to be implanted and to function in vivo;
- identification of optimal dynamic culture parameters (oxygen and carbon dioxide concentration, pH, glucose concentration, electro-mechanical stimuli) to encourage the formation and growth of engineered cardiac tissue.

5.3.6. Project statistics

The detailed description of work and the GANTT diagram are not reported here. Nevertheless, the PROBING project involves 36 person months; Table 27 and Table 28 show the total cost and financial coverage plan of the project. 50% of the project will be financed by Italian Ministry of Economic Development.

Table 27: Detail of PROBING costs.

Annual costs (sustained exclusively by the University proposing the Project)	
Internal human resources of the Italian Universities: Full Professor No. 2 x No. 3 months (POLITO + BIOTEC) Associate Professor No. 1 x No. 3 months (POLITO)	€ 64,500
Recruiting temporary collaborators: Research fellows No. 3 x No. 27 months	€ 52,000
Food, accommodation and travel expenses	€ 11,000
Purchase of consumables (including company financing for €60,500)	€ 111,500
External consultations excluding those for preparation or coordination of the project	€ 6,000
Participation in conferences/seminars pertaining to project activities	€ 3,000
Advertising/publications	€ 2,000
Total cost of the Project	**€250,000**

Table 28: Financial coverage plan of PROBING project.

FINANCIAL COVERAGE PLAN				
SOURCES		**INTERNAL HUMAN RESOURCES**	**FINANCIAL RESOURCES**	**TOTAL**
FINANCING ITALIAN UNIVERSITY (50%)		64.500		64.500
	Italian Entrepreneur Partners		60.500	60.500
PUBLIC CO-FINANCING (50%)	**ICE**		125.000	125.000
	Other public administrations:			0
		64.500	185.500	250.000

5.4. ProVaDeRiVaSa

5.4.1. Project partners' list

- BT – Biomedical Tissues s.r.l.
- Laboratori Biomicron s.r.l.

5.4.2. Project detailed background

Atherosclerosis is a multifactorial degenerative disease that affects medium and large caliber arteries. In Italy and in many other countries around the world it represents a major health problem, mostly related to a lifestyle typical of industrialized societies, and is in fact the main cause of mortality and morbidity in the developed world. One of the most practiced surgical remedies for this disease is the bypass which involves replacing a clogged tract of artery with vessels taken from the patient or with synthetic implants. Autologous arteries or veins are the most commonly used substitutes in coronary or peripheral bypass surgery, but unfortunately autologous vessels are not available in approximately 90% of patients, due to vascular disease or previous surgery. The synthetic prosthesis used to date (Dacron and e-PTFE) have not always given good results, especially for small vessels, in which restenosis of the vessel always occurs. For this reason research is directed towards the development of new substitutes for synthetic implants. The ideal replacement vessel must in fact be non-thrombogenic and have biomechanical characteristics similar to those of native vessels, a longer duration than the patient's life expectancy and no unwanted biochemical reaction. Recently, modern tissue engineering techniques have been applied in an attempt to develop vascular grafts. The general approach on which tissue engineering is based is to initially sow functional cells on biodegradable scaffolds, which will then be cultured in vitro and then implanted in vivo. The first key factor for tissue engineering is therefore represented by the choice of the scaffold. The three-dimensional structure of the scaffold serves in fact to provide support to sustain the growth of cells, their migration, differentiation and the secretion of extracellular matrix (ECM), so as to direct the formation of new tissue in the regeneration process. The term scaffold means in fact a three-dimensional porous support made of a biocompatible and biodegradable material to which the initial cells will adhere, subsequently grow until the tissue is formed and that will biodegrade at a rate similar to that of growth. At present different types of materials have been used in vascular tissue engineering, including natural proteins such as collagen, elastin and fibronectin; synthetic biodegradable polymers such as polyglycolic acid (PGA) or polylactide-co-glycolide (PLGA); decellularized vessels. Synthetic scaffolds have the advantage that they can be produced on an industrial scale in a reproducible way and that they have biochemical and mechanical properties that can be determined when being produced. On the other hand, synthetic scaffolds have the disadvantage of not possessing the essential factors that allow adhesion, migration, proliferation and differentiation of cells which are otherwise naturally present on matrix of biological origin. In particular, the use of decellularized vessels in this context is a particularly innovative approach. Regarding the choice of the most suitable cell, the ideal cells must be non-immunogenic, functional, easy to isolate, cultivate and expand in culture. For this reason many research groups have therefore focused on using autologous stem cells as a source cell. The objective of this research project, with a duration of 24 months, will therefore be the development of technologies aimed at developing vascular grafts from decellularized animal blood vessels using undifferentiated and/or differentiated mesenchymal stem cells isolated from bone marrow. This research, which is part of a field of considerable biomedical and technological interest, will bring a significant contribution to the important problem of atherosclerosis, through the creation of in vitro of biological vascular grafts thanks to tissue engineering techniques.

5.4.3. Project objective

The first objective of this project is to develop a decellularization protocol for blood vessels that maintains the structure of the extracellular matrix intact. Once the optimal decellularization protocol is identified, the second objective will be to develop a repopulation protocol for the decellularized vessel with which it will be possible to obtain a biological vascular prosthesis in vitro. This, apart from increasing the company's degree of innovation, would open a thriving vascular grafts market which currently, with functional products, is not able to meet the high demand for organic substitutes for small caliber arteries.

Consequently, the general objectives that the company intends to achieve through this research and development project, are the following:

- improve the company's level of innovation;
- identify new markets.

These give rise to the following specific objectives:

- acquisition of new protocols;
- creation of a new product.

The following operational objectives are to be met as part of this research and development project:

- develop a decellularization protocol that maintains the extracellular matrix of the vessel intact;
- develop a protocol for bone marrow mesenchymal stem cell differentiation into endothelial cells and smooth muscle;
- develop a repopulation protocol.

Although autologous vessels remain the first choice surgical approach for the treatment of atherosclerosis, the majority of these patients are not available due to vascular disease or previous surgery, which necessitates the use of substitutes for blood vessels. The vascular prostheses currently available, synthetic or biological, developed thanks to the progress made in the field of bioengineering, have limitations, for the first formed by the need to use anticoagulation to control thromboembolism, and for the second, the duration in time related to possible structural deterioration or calcification, and till now have however given successful results only in case of replacement of large and medium-caliber vessels (> 6mm). For small-caliber prosthesis, for which there is also a greater demand, there are in fact greater problems at stake because of slower blood flow, resulting in a higher frequency of thrombosis and platelet aggregation. Recently, modern tissue engineering techniques have been applied in an attempt to develop vascular grafts, in particular biological scaffolds such as extracellular matrices using prefabricated, non-vascular decellularized ducts, and decellularized allogeneic or xenogeneic blood vessels. In particular decellularized biological scaffolds have the advantage of possessing an extracellular matrix (ECM) rich in essential factors for adhesion, migration, proliferation and cell differentiation. While on the one hand, the types of scaffolds that are more suitable to use are clearly emerging, the same cannot be said for different cell types to be planted in such support, ranging from differentiated cells such as endothelial and/or muscle cells, to autologous differentiated or undifferentiated stem cells. Up to now, the tissue engineering techniques recently introduced have only led to partial results and, when applied to native tissue matrix, are strongly influenced by prior essential decellularization procedures, which should not inevitably undermine their compositional, physical-chemical, structural, biomechanical and hemodynamic characteristics. Furthermore a major problem in the construction of an engineered replacement vessel is cell repopulation. Several studies have shown that repopulation is more efficient if performed using a bioreactor. This device makes it possible to recreate physiological conditions more similar to those that occur in the native blood vessel. Consequently with this project, BT Srl aims to solve some fundamental problems, first among which the development of a decellularization protocol that maintains the structure of the ECM integral in order to obtain a biological scaffold that does not create problems with rejection and allows the vessel to integrate properly with the surrounding organs. This will allow the company to patent a decellularization protocol to build a scaffold to be used as a support for the development of new biological vascular prostheses. Once

the optimal decellularization protocol is identified, the focus will be on developing a repopulation protocol for the decellularized support in order to obtain a biological vascular prosthesis. This will make a significant contribution to current knowledge in the field of repopulation of vascular scaffolds and will permit BT Srl to enter the thriving market of vascular prostheses.

5.4.4. Consortium detailed description

BT - Biomedical Tissues

It is a limited liability company founded in June 2008 as part of the project co-funded by the EU, P.O.R. Sardinia 2000-2006. The company has its headquarters in Sestu, Viale Monastir Km.7,300 - 09028 SESTU (CA), and operational headquarters at the Science and Technology Park of Sardinia, Building 3, Location Piscinamanna, 09010 Pula (CA). It is legally established as an S.r.l. and governed by a Board of Directors.

BT deals with research, experimentation, design, synthesis, production, manufacture, processing, use, and marketing in the public and private sectors, cell cultures of any type and size of an innovative nature, and their bioreactors, for the tissue engineering and regenerative medicine sector, or for other high-tech sectors. In particular, the company deals with the development of protocols for the expansion of static or dynamic media, whether of cartilage cells (chondrocytes), or adult stem cells taken from patients with joint damage. It also takes care of the removal/expansion and differentiation of mesenchymal stem cells for different fields of application, ranging from regenerative medicine to the development of new tests for acute toxicity in vitro. In the last year research activities have also focused on the development of new protocols for decellularization and repopulation of blood vessels to the targeted development of new biological vascular prostheses. As part of this research project the activities carried out by BT Srl specifically concern the development and application of a decellularization protocol; the phenotypic collection, expansion and characterization of mesenchymal stem cells to be used for subsequent repopulation, and to develop a repopulation protocol, using the culture device developed by Biomicron Srl.

Laboratori Biomicron Srl

It was founded in October 2006 as a start-up of Biomicron Srl already present on the market since 1978.

The company is located in Corso Casale 52 - 10153 Turin, with operational headquarters in Via Lessolo 19 - 10153 Turin.

Its core business can be identified in components and accessories for in vitro diagnostics. Its main activities concern the production of disposable products for in vitro diagnostics and allergology, production of surgical instruments to support artificial cardiac valve grafts, design and manufacture of laboratory instruments.

Current and previous experiences include:

- design and production of a hip prosthesis, with improved mechanical profile, verifying the use of multi-layered materials (carbon - Polyeterimmide) coated with biocompatible technopolymers (in collaboration with the University of Rome Tor Vergata);
- design and manufacture of systems for detecting the BAC (blood alcohol content) in collaboration with the San Luigi di Orbassano Hospital (Turin);
- development of techniques and production of kits for evaluating the pharmacokinetics of chemotherapeutic agents through HPLC;
- design and manufacture of radio frequency identification systems (RF-ID) for diagnostic purposes;

- design and installation of industrial fermenters for bread-making.

Currently being developed within the company:

- devices for allergy diagnostic based on lab-on-chip techniques;
- advanced implantable sensors;
- polymeric implantable devices;
- bioreactors for tissue engineering

Laboratori Biomicron Srl brings its expertise into the field of customized production of laboratory instruments. Currently, other laboratory equipment is being developed in the company similar to that of interest for the ProVaDeRiVaSa project, including bioreactors for tissue engineering, mostly in cooperation with the Polytechnic of Turin, the University of Turin (Center for Molecular Biotechnology) and the Imperial College London.

5.4.5. Expected results

With reference to each operational objective, the achievement indicators are shown in schematic form.

Development of a decellularization protocol that maintains the extracellular matrix of the vessel intact.
What indicators can be considered:

- comparison between control vessel not subject to decellularization and decellularized vessel, using immunohistochemical techniques and mechanical tests.

Development of a protocol for bone marrow mesenchymal stem cell differentiation into endothelial cells and smooth muscle.
What indicators can be considered:

- comparison between endothelial cells and primary smooth muscle and differentiated mesenchymal stem cells using immunofluorescence techniques and analysis of gene expression.

Development of repopulation protocol
What indicators can be considered:

- comparison between control vessel not under decellularization and repopulation and repopulated vessel, using immunohistochemical techniques and mechanical tests.

The performance indicators for each specific objective are reported in schematic form:

Acquisition of new protocols
What indicators can be considered:

- Patenting of protocols.

Realization of a new product
What indicators can be considered:

- patenting of protocols;
- increase in sales due to access to new markets.

The actions to be taken to protect the results achieved during the development of the project can be simply summarized in presenting international patents related to the processes/products developed. These results

will also be disseminated by publications in international scientific journals. However, with regards to the exploitation of research results, a widespread marketing policy will be used bent on the sale of patents and transfer of the proprietary know-how acquired.

5.4.6. Project statistics

The detailed description of work and the GANTT diagram are not reported here. Nevertheless, the ProVaDeRiVaSa project involves 21 person months; Table 29 and Table 30 show the total financial plan of the project and its analytical cost plan. 75% of the project will be financed by Regione Sardegna.

Table 29: ProVaDeRiVaSa financial plan.

Proponent	Project cost	Contribution requested	% Contribution requested	Proponents' share
BT – Biomedical Tissues	90.000	67.500	75	22.500
Laboratori Biomicron	70.000	52.500	75	17.500
Total	160.000	120.000	75	40.000

Table 30: Analytical project cost plan.

Cost Category	Description	Amount (Euro)
Staff	Employees (No. man days) 147 Non employed staff (No. man days) 440	78.595,75
Tools and equipment	Milling and molding devices, laboratory equipment, computer equipment (depreciation)	5.000
Third-party services	Development of test benches and control software (Consultant EnginLife – Torino) Consultancy for modeling and mechanical testing of scaffold	20.500
Other project costs	Engineering plastics, metals and molds, reagents for cell culture (culture media, growth factors, antibiotics, etc.), disposable material (pipettes, petri dishes, pipette tips etc.), material for	36.904,25

	immunohistochemistry (antibodies, histological staining)	
General expenses	Facilities rental, electrical and telephone utilities, travel, office supplies, administrative staff	19.000
Total Project Cost		**160.000**

5.5. BIOBONE

5.5.1. Project partners' list

- Laboratori Biomicron s.r.l. (BIOMICRON)
- Camerson S.p.A. (CAMERSON)
- Politecnico di Torino – Department of Material Sciences and Chemical Engineering (POLITO)
- Università di Torino – Department of Biomedical Sciences and Human Oncology (UNITO)

5.5.2. Project detailed background

The replacement and regeneration of bone tissue has been studied for a long time, as it affects mainly orthopaedic surgery, neurosurgery, reconstructive surgery and dentistry. According to recent estimates, approximately 7.9 million fractures are treated in the U.S. alone each year. Among these treatments, approximately 1.5 million bone grafts of different types are performed, with the final goal to improve and speed up the solidification of fractures of long bones and to increase skeletal reconstruction in the treatment of maxillofacial defects.

In this scenario, regenerative medicine has becomen more and more the solution to bone substitution by production of tissues using autologous cells, i.e harvested from the patient who is also the procedure recipient. In this way, it is posible to regenerate damaged anatomical parts that may express features identical to physiological tissues. In fact, currently bone substitute gold standard is represented by autologous bone tissue, as it is characterized by good mechanical properties, it is osteoinductive, osteoconductive and free from immunogenic risk.

However, autologous bone grafts are still hindered by high absorption rate, potential risk of local infection, morbidity on donor sites and reduced availability.

Aiming at overcoming the still existing limitations in the use of autologus tissue in bone substiuttion and regeneration, a novel technology is herein proposed which is base on the proper combination of cell response to electrical stimuli (to obtain a controlled reaction and cue cell organization) with advanced cutting-edge cell culture methods (through the use of bioreactors). The main application of such technology has generated a device able to improve the production of autologous tissue grafts.

5.5.3. Project objective

The BIOBONE project aims to innovate the product "bioreactor" for bone tissue engineering using a new conceived biosensor/bioactuator system for real time stimulation and monitoring of the cell culture. More in detail, thanks to the continuous monitoring of several parameters such as the adhesion of cultured osteoblasts to the surface onto which they are seeded and the stress they withstand within the bioreactor,

appropriate stimuli can be provided to properly modify culture conditions in a driven-by-cells-response loop.

This result can be achieved by recording data furnishing the most complete information on the status of the cell culture and, using a tailored software control system, to produce an immediate closed loop feedback from the bioreactor, according to the state of the culture and to the effect required.

The innovation proposed has an international relevance, since other bioreactors supplying the same features are not present on the market.

The project will lead to the pre-industrialization of a high competitive product.

Bioreactor

The device for osteoblasts culture incorporating the biosensor/bioactuator system developed in the project BIOBONE will consist of two main parts: (i) the culture unit, hosting the biosensor/bioactuator system on which osteoblasts will be seeded; (ii) the control unit, that will gather and process data measured in the culture unit environment and will set/modify the culture conditions properly.

The culture unit hosts the biosensor/bioactuator, it is filled with culture medium and keeps suitable environment condition to make cells survive, proliferate and differentiate.

A set of biosensors interfaces control and culture unit, measuring the environmental parameters that cells withstand during the culture.

The control unit is designed in order to gather and process data collected by the biosensors in the culture unit and, based on the recorded information, to give an appropriate feedback to cells (in terms of maintainement/variation of the culture conditions) through the bioactuator. The data gathered by the control unit are used to manage the culture parameter coming from the culture unit in a feedback loop (as already shown in Fig. 156, paragraph 5.3.3. about the PROBING project).

Having a real-time measure of the culture parameters (related to the functional state of cultured cells) will make the bioreactor a sophisticated test instrument.

The new culture device technical specifications will be based on bottom-up procedures focused on the culture chamber, mainly considering culture requirements for osteoblasts and analyzing the final user's need.

Culture unit

The culture unit (Fig. 157) consists of a culture chamber and a culture medium circulation system.

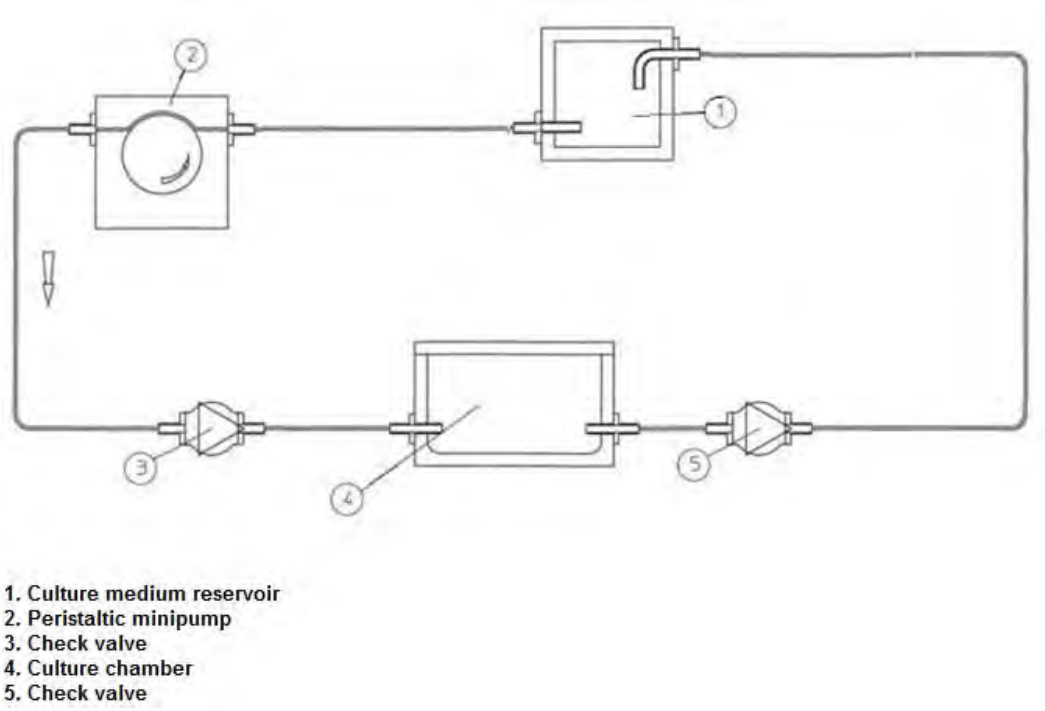


Fig. 157: bioreactor culture unit diagram.

The miniaturized culture chamber will host the biosensor/bioactuator system, sized 5x5mm. A chamber of 200µl internal volume will be small enough to allow culture medium saving, given culture medium relevant cost.

The low-flow culture medium recirculation system (less than 1ml/min) will consist of a peristaltic minipump (Fig. 158) and by special silicone tubes able to ensure gas exchange between the incubator environment and the culture medium in order to keep the expected gas rate (5% CO_2) in the culture medium itself.

Two check valves will hamper fluid reflux when the circuit is put off.

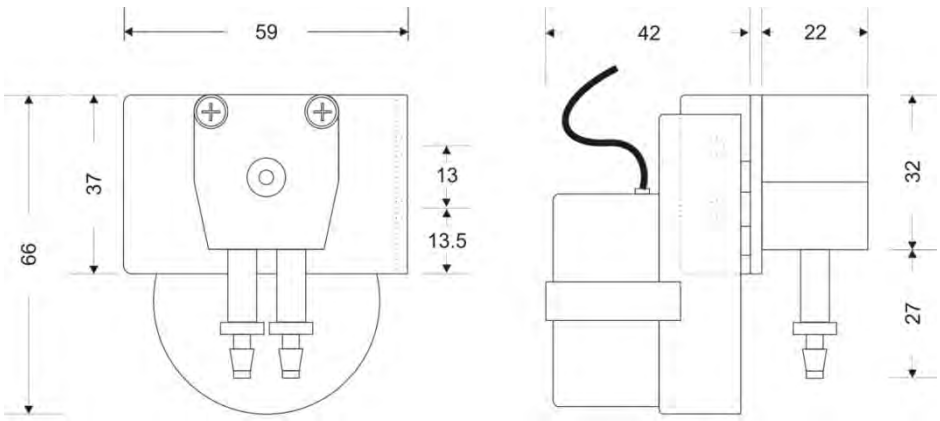


Fig. 158: Layout of the peristaltic minipump in the bioreactor recirculation system.

The culture chamber will be equipped with an interface hosting the environmental sensor probes.

Appropriate disposable minisensors will be specifically developed in order to measure chemical parameters (pH, oxygen rate, carbon dioxide rate, glucose and lactate rate) and physical parameters (temperature). A

wall microconnector will be used to transfer sensors data to the control unit and to supply controlling signals to the biosensor/bioactuator system.

The culture unit will be designed in order to ensure full sterility once closed and activated. To reach this aim, materials and components of the culture unit will be properly selected in order to be autoclavable, thus allowing a quick and cheap sterilization.

Biosensor/Bioactuator

A fundamental component of the proposed culture device and the MOST IMPORTANT INNOVATION of the BIOBONE project is the biosensor/bioactuator system, which is able (i) to measure the magnitude of the force exerted by the cells during their adhesion on the surface of the implant, and (ii) to provide mechanical stimuli to the cells in order to promote their adhesion and proliferation properly. This device will be integrated in the culture device, and will also permit to monitor the cellular adhesion process in real time, thus allowing to obtain a deeper knowledge of the osteointegration process.

The possibility given for monitoring, thanks to this component, the adhesion force of cells is fundamental to get more insight into the understanding of the mechanisms involved in the osteointegration on implant surfaces.

The real time, in situ monitoring of the process of cellular adhesion is a challenging topic. Nowadays, information about the osteointegration process are obtained by stopping the experiment, by acquiring images of the cellular morphology and by performing different biological tests on the immobilized cells. This approach, even if provides complete data on the cellular properties at a specific time point of the osteointegration process, does not allow the monitoring of the whole dynamics of the process. Moreover, the real time survey of the cellular adhesion can be useful to identify the more appropriate time points to provide cells with electro-mechanical stimuli for guiding their culture properly.

The biosensor (that senses the adhesion force) and the bioactuator (that provide the electro-mechanical stimulation) are embedded in a compact device. This solution can be realized using capacitive compact structures (with areas of few mm2), manufactured by adopting a thick film deposition technology and by using biocompatible materials such as titanium and ceramics. Once the cells adhere to the surface of the structure, the mechanical force that they exert causes a reaction force from the material and an overall variation of the electrical impedance of the structure,as a consequence. This variation can be easily measured by applying to the structure a sinusoidal voltage, and by recording the current that passes through the structure, obtaining the relative impedance spectrum. As the process of cellular adhesion can lead to relevant variations on the impedance spectrum, the impedance spectrum can ultimately be correlated to the cellular adhesion force.

Control unit

The control unit will consist of four main blocks: the first block will perform analogical signal conditioning, in order to acquire signals such as temperature, humidity, voltage and current; the second block will be a programmable waveform and frequency signal generator for impedance measurement; the third block will have the role of local user interface with LCD display and a keyboard, and it will be also the remote interface via serial line RS232 and/or USB; the fourth block will play the role of control unit for the other blocks and will consist of a DSP with processing power and memory suitable for the application.

For an easier use and management not only of the of the culture device but also of the acquired and processed data, a dedicated PC software will be developed with a user-friendly graphical interface, by

means of which the operator will easily set and monitor the cell culture. All the project choices will be step by step validated, in order to identify and to resolve any technical defect, for reaching the optimal final version of the culture device prototype.

The BIOBONE project aims at developing an equipped, closed loop cell culture device useful to research laboratories, and that goes beyond the limitations of current commercially available products. In case of promising results, this study could lead the proponents to initiate a patent procedure for the realized device.

As already assessed, limited commercial progress achieved by tissue engineering products by now are due to almost fully manual production processes that (i) are very costly if considered in a large scale production perspective and (ii) have high contamination probability and lack in homogeneity.

So, a standardized, closed operator-independent production process, using automatic production steps based on bioreactors, is cheaper, safer and more compliant with regulations.

Despite high development costs, this approach will improve the cost-benefit ratio of this manufacturing process, even favouring its industrial scale-up.

But an operator-independent cell culture needs a deep knowledge of the processes of cell proliferation, differentiation and, in the case of this project, osteointegration.

So, in order the bioreactors to reach a large diffusion, it is of primary importance to build bioreactors able to work through closed-loop mechanisms, to sense the variation of signals coming from cultured cells and to act on the culture to drive its behaviour.

Because of the lack of a relevant number of cost-benefit analysis in a clinical context and of a relevant statistical sample, it is difficult to plausibly define the remuneration probability related to the production and implantation of tissue engineered products, and this heavily influences the consistence of business plans based on the diffusion of tissue engineering.

Since this is a very critical economic model with a very unstable bioreactor demand, the reason why industry conservatively invests in development of bioreactor production technologies and slowly undertakes bioreactor commercialization.

A closer interaction between academies and industries may favour more effective and cheaper bioreactor design and production with respect to the ones currently distributed by SMEs and, to a lesser extent, by big industries, and it may drive to the development of bioreactors able to supply a complete picture of the state of the ongoing culture.

Innovating the product “bioreactor” for bone tissue means also identifying culture processes, optimal culture parameters to be monitored and kept in order to standardize tissue engineered products production and to supply quality and traceability, through maximizing the information obtained from the culture and minimizing the response time over the culture itself.

So, the development of more “intelligent” bioreactors has for industry a further advantage of carrying out easier production strategies, facilitating their design, limiting the amount of technical specification and, as a consequence, reducing production and operating costs.

Bone tissue bioreactor market is at an early stage in Europe and it is a very interesting niche, taking advantage from the growth of tissue engineering market and limited competition.

Thus, a focused innovation in this field, mainly if protected by IP strategies, can give the industries that runs this path significant advantages.

At the state of the art, several already assessed technologies exist, that are based on the measurement of a variation of impedance in order to monitor cell adhesion on a substrate.

A prior art search identifies some devices using this principle; the most significant of them are:

- US Patent 7732127 (2010), Wang et al., "Dynamc monitoring of cell adhesion and spreading using the RT-CES system"
- US Patent 7470533B2 (2008), Xu et al., "Impedance based devices and methods for use in assays"
- US Patent 7459303 (2008), Wang et al., "Impedance based apparatuses and methods for analyzing cells and particles"

Some commercial devices (xCELLigence System della Roche Applied Science) are also on the market.

Nevertheless, the most part of this systems is not specifically oriented to analyze osteoblast adhesion on implants, but to real-time analyze “in vitro” cell culture.

Usually, these devices use a system of interconnected electrodes under the culture substrate.

Nowadays it is not possible to find on the market a miniaturized integrated system able to supply ad hoc electrical stimulation to cells put straight in contact with the device and, at the same time, their adhesion real-time monitoring.

5.5.4. Consortium detailed description

Laboratori Biomicron (BIOMICRON)

Core business: components and tools for in vitro diagnostics.

Field of activity: manufacturing industry for transformation of plastics and metals.

Main production activities.

- production of disposables for in vitro diagnostics;
- production of disposables for suppor of surgical implantation of artificial heart valves;
- production of allergologic devices;
- design and development of laboratory instruments.

Camerson S.p.A. (CAMERSON)

CAMERSON S.p.A. works in the field of electromagnetic components and electronic devices for industrial, civil and transport sectors; moreover, it is involved in photovoltaic power plants for industrial application

Main production activities:

- safety transformers EI30 for electrical and electronic application for power in the range of 0,6 – 2,8VA. Its market is based on the companies that design and produce electrical and electronic devices in any sector (white and brown goods, industrial electronics, telecommunications…);

- integrated power supplier for electronic application and for LED lights;
- Inverters for fluorescent lamps and railway application;
- Integrated systems for railway carriages.

Politecnico di Torino – Department of Material Sciences and Chemical Engineering (POLITO)

Politecnico di Torino takes part to the BIOBONE project involving the Materials and Microsystems Laboratory (ChiLab), part of the Department of Material Sciences and Chemical Engineering (DISMIC).

ChiLab's activities involve different areas of scientific and technological research, as::

- study of innovative nanostructured coating and surface treatment for microelectronic, mechanical and biomedical application;
- design and realisation of microsystems for electromechanics, microfluidics and biomedical sensors;
- basic study of innovative materials.

Its laboratory equipment involves:

- systems for advanced characterization of materials and nanostructures (FESEM, XPS, AFM, SNOM, UV-VIS, FTIR and Raman spectroscopy, OCA, XRD);
- advanced systems for delivery of nanostructured films and surface modification of materials (LPCVD, RF-PECVD, ECR-PECVD, RF-Magnetron Sputtering, Plasma Polymerization, APP, plasma etching);
- systems for realisation of micro and nanostrucures (UV and e-beam lithography, RIE).

Università di Torino – Department of Biomedical Sciences and Human Oncology (UNITO)

The Department of Biomedical Sciences and Human Oncology (DSBOU) of Università di Torino includes more than 50 people working in the field of Pathologic Anatomy, Dermatology and head-neck pathology. Inside the DSBOU, the Dental School works as Department of Odontoiatry of S. Giovanni Battista Hospital (third hospital in Italy) and as Didactic and Research Department of Università di Torino. The DSBOU includes 20 laboratory, and it is equipped with the tools needed to carry out biological, histological and histochemical analysis.

5.5.5. Expected results

The bioreactor developed in the BIOBONE project will allow continuous monitoring of the cell adhesion force on a surface, also supplying electro-mechanical stimuli (see paragraph 2.1) forcing the surface with an electrical signal. As a final result, the developed product will be an useful tool for laboratories and research centres in order to more deeply study the process of osseointegration. The research of correlation between cell behaviour (rate of growth and differentiation, rate of cytokines released in the culture medium and so on) and different electro-mechanical stimulation patterns (variation of intensity and frequency of stimulation) will improve the study in vitro of mineralized tissues, so upgrading the knowledge of bone biology.

The product developed finds its market in university research centres, hospital research centres and red-biotech companies, so having a potential market of about 500 customers in Italy and more than 10.000 all over the world.

The BIOBONE project will drive to:

- patents on the technology of bioreactors;
- pre-industrialization of a high competitive product;
- scientific publication on relevant journals in the field of bone technology and tissue engineering (e.g. Bone, Journal of Bone and Mineral Research e Plos One), in the field of materials for biological application (Biomaterials, Acta Biomaterialia) and in the field of sensors (Sensors and Actuators B, Biosensors and Bioelectronics).

5.5.6. Project statistics

The detailed description of work and the GANTT diagram are not reported here. Nevertheless, the BIOBONE project involves 63 person months; Table 31 shows the BIOBONE analytical cost plan. 60% of the project will be financed by Regione Piemonte.

Table 31: BIOBONE analytical cost plan.

Partner	BIOMICRON	CAMERSON	POLITO	UNITO	Total
Staff	€ 86.223,90	€ 98.000,00	€ 32.000,00	€ 38.000,00	€ 254.223,90
Tools and equipment	€ 27.489,14	€ 7.800,00	€ 0,00	€ 0,00	€ 35.289,14
Consumables	€ 32.560,96	€ 11.000,00	€ 26.000,00	€ 27.950,00	€ 97.510,96
Third-party services	€ 27.800,00	€ 45.500,00	€ 5.000,00	€ 5.000,00	€ 83.300,00
Other project costs	€ 10.800,00	€ 4.700,00	€ 15.000,00	€ 7.050,00	€ 37.550,00
Management costs	€ 10.726,00	€ 3.000,00	€ 0,00	€ 0,00	€ 13.726,00
Total/partner	€ 195.600,00	€ 170.000,00	€ 78.000,00	€ 78.000,00	€ 521.600,00

6. Bioexpansys – Cell Expansion Devices. Spin-off and start-up: a proof of concept

6.1. Executive Summary

The advances in medical research in recent decades have led to explore fields that seemed unimaginable just a generation ago, creating huge expectations concerning the possibilities of medicine in the years to come. One of the hot topics of the last decade concerns stem cells and the ability to create or regenerate tissues and organs in the laboratory.

In this regard, it should be noted that the Italian biotech sector is of recent establishment and is in continual evolution, with the majority of companies having been founded in the late '90s and early 2000s and currently there are approximately 500 public and private structures. The future of regenerative medicine and related technological innovations looks promising and high growth rates are expected, both in the number of companies as well as in their investments, over the next few years. In particular, significant growth opportunities related to therapies involving the need for transplants are foreseen, since tissue engineering technology may also be able to play a decisive role in bridging the gap of the current shortage of organs.

The tissue engineering bioreactors are tools allowing in vitro tissue and organ culture, aiming at supplying laboratory models for tests and at producing tissues and organs to be implanted in patients.

Bioexpansys (Cell Expansion Devices), the company subject of this business plan, intends to position itself precisely in this sector at the frontier of scientific research, designing and developing bioreactors for dynamic cell culture; these are indispensable technological devices for the creation of engineered tissues in the laboratory from patient cells. Initially it will offer a bioreactor for cardiovascular, epithelial and muscle tissue, characterized by incremental innovation of performance compared to its competitors, a high level of automation and a competitive price since it is precisely this latter factor which appears to be a major driver for the large-scale deployment of this type of product in hospital and research facilities.

The strategy will be divided into two sequential phases: a first phase of product development and industrialization, followed by production and marketing.

The company will keep bioreactor design and assembly functions inside the company and train a small network of specialized consultants and vendors in order to ensure the customer both the necessary technical skills for technology consulting as well as negotiating skills to close the sale. Outsourced production will be entrusted to an industrial partner with over 30 years of experience in the production of basic biomedical components which will also play the role of co-financier of the fledgling company.

Compared to the competition, the company has a definite price advantage (the product will cost approx. 55,000 € in the basic version, providing a reduction in purchase costs of up to 50%, with the same characteristics, compared to competitor products) and a relative time advantage, given that on the market today there is neither a dominant design nor a very large number of established competitors. Among the innovative technical features of the product under development, the possibility of expanding cells of different tissues and the use of innovative materials and disposable items to bring down production costs are worthy of mention. Moreover, the slim corporate structure will facilitate a more direct and personalized approach with the customer, offering specific additional services considered interesting on the basis of market research carried out.

The benefits which might accrue from the use of such technological innovations are undeniable, such as:

- increase in process quality and safety standards;

- decrease in biological risk factors and production costs;
- process accessibility by potential manufacturers (e.g. hospitals and cell/tissue banks), resulting in sector market growth and competitiveness.

In addition to the product characteristics, the success of the company will also derive from the technical quality of the entrepreneurial team. The founders are three researchers from the Politecnico di Torino with proven experience in the field of biomedical and mechanical engineering, fundamental skills for the technical design and development of bioreactors; currently they are actively working with the Politecnico di Torino, the University of Turin and Imperial College London. Moreover, experience in the commercial and administrative field of one of the team members is another strong point of the future enterprise, since it brings managerial skills fundamental to the survival of a startup.

The target market of Bioexpansys is international. For strategic reasons related to the geographic proximity of potential partners and easier customer accessibility, the market analysis is currently focused on Italy.

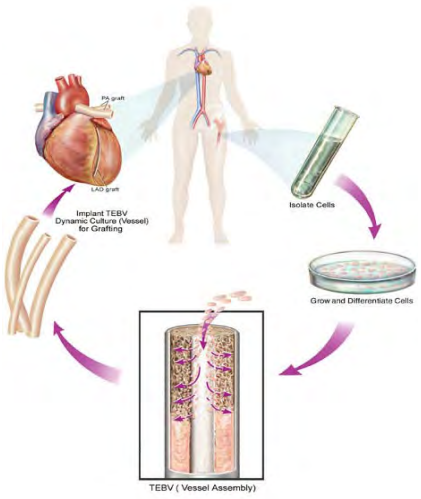
The team has the objective of being on the market within 3 years and to install products within 5 years in 10-20% of Italian research facilities. This analysis does not take into account hospitals as target customers since it is expected to reach these over a longer time frame than that of the forecast horizon of this business plan.

The investment required for this project is estimated to be between **420,000 €** and **640,000 €**, divided into 250,000 € in the first two years for the development phases and the remainder necessary for the subsequent marketing phase. Part of the initial funds could be covered by public tenders and by shareholders of the company. According to the forecasts and objectives, the company believes it can achieve turnover in the 5th year of operation (3rd year of sales) of approx. **3.6 M€**.

6.2. Venture Description

Bioexpansys (Cell Expansion Devices) will be founded in September 2011 in Turin, with the objective of producing engineered and automated devices aimed at improving the cultivation of damaged human tissues using the patient's own cells (for this reason we speak of autologous tissues).

The devices, called bioreactors, are tools closely related to the field of tissue engineering (a field of biomedical engineering) and research carried out in recent years on adult stem cells has opened new horizons regarding their use in regenerative medicine.



Bioreactors are dynamic devices capable of *mimicking* the chemical and physical conditions inside a living organism to recreate tissues and organs in the laboratory.

Procedure:

- cells are harvested from the patient;
- the harvested cells are seeded into an artificial support (scaffold) and placed in the bioreactor;
- at the end of the culture (after approx. 20 days), the tissue or organ is ready to be transplanted into the patient to replace that damaged.

Bioreactor target customers can be divided into two main categories:

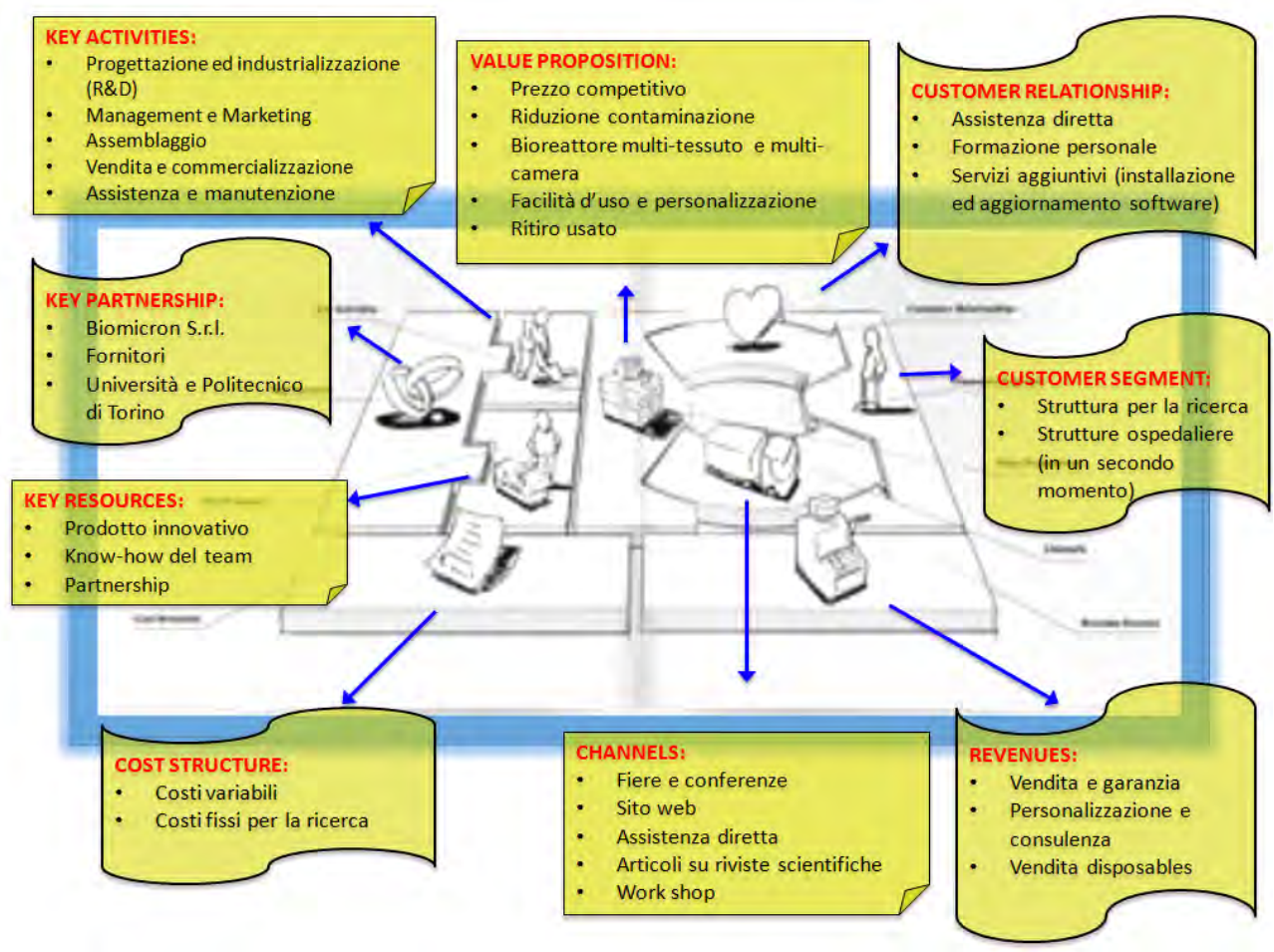
- hospitals;
- university and clinical research centers.

Since in Italy the practice of reproducing damaged organs and tissues is not yet completely rooted, the company has as its first objective that of providing bioreactors able to support their activities to research centers.

Subsequently, with the development of procedures which provide for the creation of organs in vitro, the company will also be in a position to address the first segment mentioned.

6.3. Business Model

The business model, i.e. the operating logic of the future enterprise, has been structured using the nine essential cornerstones of "Osterwalder methodology"; "ANNEX II - BUSINESS MODEL" contains a brief description of each item.



KEY ACTIVITIES:

- Design and industrialization (R&D)
- Management and Marketing
- Assembly
- Sales and marketing
- Support and maintenance

KEY PARTNERSHIPS:

- Biomicron Srl
- Suppliers
- University and Polytechnic of Turin

KEY RESOURCES:

- Innovative product
- Team know-how
- Partnerships

COST STRUCTURE:

- Variable costs
- Fixed research costs

CHANNELS:

- Exhibitions and conferences
- Website
- Direct support
- Articles in scientific journals
- Workshops

REVENUES:

- Sales and warranty
- Customization and consulting
- Disposable sales

CUSTOMER SEGMENT:

- Research facilities
- Hospitals (at a later stage)

CUSTOMER RELATIONSHIP:

- Direct support
- Personnel training
- Additional services (software installation and updating)

VALUE PROPOSITION:

- Competitive price
- Contamination reduction
- Multi-tissue and multi-chamber bioreactor
- Ease of use and customization
- Trade-ins

6.4. Customer Value Proposition

The engineering knowledge acquired over the years by researchers at the Politecnico di Torino have made possible the design and implementation of new scientific discoveries in the field of instrumentation, as in the case of bioreactors.

Bioreactors for dynamic cell cultures are instruments designed to ensure appropriate culture conditions (e.g. pH and temperature), apply mechanical stress and electrical and chemical stimuli to cells to provide conditions as close as possible to physiological conditions. To obtain quality functional engineered fabrics, promote cell differentiation toward the phenotype sought and to encourage generation of a functional tissue, it is fundamental to "mimic" the conditions and stress that cells "experience" in the native environment.

From the technological point of view, the product being finalized by researchers is primarily characterized by LVDT linear actuators able to reach high power and speed, producing, as a consequence, high stimulation amplitudes and frequencies. Secondly, it is a device which can cultivate several types of tissue/cell using a single culture chamber, reducing time, cost and improving the quality of the final product. The use of disposable technology and economic and commercial locking/unlocking systems make it possible to offer modular and scalable products, providing the dual benefit of reducing contamination risk and making the purchase price more competitive.

Parameter and process monitoring electronics using microchip-based circuits is accompanied by a software package developed using the commercial platform LabView® which automates the culture parameter and process system in a closed loop, providing as output data analysis in real time. The support software used, at the same time simple, user-friendly and comprehensive, also allows users with lower IT skills to operate directly on the culture. In any case, increased automation simplifies end user operations, reduces the time dedicated to control and modification of culture parameters and, last but not least, simplifies the process.

All this reduces the risk of accidental errors associated with manual parameter changes and unintentional contamination since the operator does not need to directly intervene in the culture chamber.

Furthermore, the spin-off intends to provide additional sales services such as trade-ins, upgrades and after-sales support including remote updates of control software and, if required, installation and training of the computer package (In "ANNEX III - Value proposition" see the value proposition table).

To determine whether the proposed new Bioexpansys product is indeed deemed by the market to be valid and effective, direct primary research was carried out (interviews and questionnaires); in the next section the results obtained from potential interested parties are illustrated.

6.4.1. Questionnaire Analysis

In order to evaluate the effectiveness of the value proposition proposed by Bioexpansys, a questionnaire was designed and sent to understand the preferences of potential bioreactor users. 19 responses were received in total, corresponding to approx. 23% of questionnaires sent. Respondents belong to public and private research centers (27%), universities (68%) and, to a smaller extent, hospitals (5%).

Thanks to the information obtained from questionnaires and secondary analysis, it was possible to identify five main Italian biotechnology market demands associated with the product. We will now turn to a specific analysis of the data obtained and the most important considerations on the various characteristics. The full results of the questionnaires can be found in "ANNEX IV – Analysis of questionnaires".

Competitively priced product

Approx. 85% of responses identified as “Fundamental” (42%) or “Very important” (42%) the reduced cost of purchase of bioreactors, with an average score of importance of 4.28 out of 5. In this regard, the Bioexpansys product will reduce purchase costs for end customers. Current competitors offer products with prices ranging between 50,000 and 350,000 euros approx.; the Bioexpansys sales price range is positioned at the lower end of this range, between 50,000 € (basic model) and 70,000 € (model complete with all additional options), but with characteristics similar or superior to competing models in a price range between 80,000 € and 120,000 €, thus ensuring the customer a saving of up to 50%. The reasons for the lower prices are associated with the use of disposable components, lower development costs and the use of other innovative materials; the entrepreneurial team “will exploit” previous design knowledge and research in a university context to reduce research costs which competitors include in full in the final sales prices.

Multi-tissue and multi-chamber Bioreactor

The Bioexpansys bioreactor will be a multi-tissue (a single culture chamber suitable for multiple tissues) and multi-chamber (parallel culture chambers to perform more experiments) product. The benefits are savings in purchase cost, given that there is no need to change device to work on a new tissue, and savings in culture times (to work on a different tissue, a new bioreactor with a single chamber costs between 50,000 € and 150,000 €). For those who purchase a bioreactor for the first time, use could increase culture quality and save on personnel costs for both in the monitoring as well as the culture management phase; with an average hourly cost of 20 €, routine culture operations can account for up to 2-4 hours per day during the entire duration of the culture (approx. 21 days), resulting in a cost attributable to monitoring and modification of 1,200 € per culture. Assuming 5-10 cultures per year performed with 3-4 parallel cultures, as a first approximation a saving of around 20,000 € per year could be obtained on these items.

Reduction in contamination risk

Contamination in a cell culture can occur whenever the operator intervenes on the cultivated tissue in any of the experimental phase (seeding, cultivation or extraction phase). According to the questionnaires, the average score of risk importance attributed to phases varies between the category of users or non-users of the bioreactor, reaching peaks of 2.15 out of 3 in the extraction phase for the former and 2.1 out of 3 in the seeding phase for the latter. In general, the interest in reducing contamination risk is high in both categories and confirms the orientation of the market towards more automated products equipped with *disposable elements,* such as those offered by Bioexpansys, which allow contamination risk to be reduced due to less human intervention. In addition, risk reduction is facilitated by use of an automated pumping system which allows replacement of the culture fluid without opening the culture chamber. The combination of all these solutions can technically reduce overall contamination risks by approx. 90%.

Ease of use and customization

To complete a cell culture, on average, an operator must measure many parameters, modify them should they be outside the assigned range and replace the culture liquid on average 1 to 2 times a day for 21 days.

With the bioreactor, data processing, parameter control and liquid replacement will be carried out automatically and monitored by the software, providing greater safety and reliability of results and a reduction in both operator stress and human error. 57% of respondents were interested in ease of use (average score 3.8 out of 5). Moreover, almost 72% of respondents would appreciate software which can be customized by the user and all current users of bioreactors define as “Fundamental” (29%) or “Very important” (71%) to be able to use such a service, also with a price differential.

Trade-in service and additional services

One of the requirements emerging from the questionnaires not be underestimated is the request to collect the bioreactor once its useful life is over; the questionnaires show that 85% of users would be interested in collection or trade-ins. The customer benefits of this service are related to the reduction in cost, de-commissioning time, in the event of substitution and not expansion of instrumentation in the laboratory and possible reduction of the purchase price for a new bioreactor. In particular, from the questionnaire data, it emerges that only 29% of current users received this service.

As for bioreactor installation and training, the totality of users declared interest in this service, even though this is already provided in 71% of cases. On the other hand, on-line, telephone and remote support is considered of little importance in 14% of cases and of the remaining 86% only half received at least one of these services. The reason why Bioexpansys intends to support its customers with these services included is therefore clear and justified.

In light of the analysis of the questionnaires received, carried out via interviews and secondary research, the value proposition proposed by Bioexpansys appears to be valid and effective.

6.4.2. Competitor mapping

In order to understand the current competitor offer on the market, using the "Quality Function Deployment" approach, a benchmark was carried out on the features most requested by the market, verifying whether the features offered by the Bioexpansys bioreactor are present in direct competitor offers already on the market.

The analysis carried out showed that the technical features proposed by Bioexpansys are not currently offered by major competitors in the industry. Via questionnaires, positive feedback on the innovative features provided by the company was obtained.

For the complete benchmark on the features of the bioreactor, see "ANNEX V - Competitor mapping".

6.5. Management

Giuseppe Falvo d'Urso Labate, one of the three founding shareholders, began to consolidate the idea of starting a business as a result of experience acquired during the European project BIOSCENT which made him perceive how fascinating and rewarding it could be to work in the field of bioreactor development and production. During work carried out together with colleagues and partners Diana Massai and Umberto Morbiducci, within the scope of other European projects in the field of regenerative medicine, the initial perception was strengthened by bringing the three to see the real possibility of bringing to the market a bioreactor of their design.

6.5.1. Founding partners: professional skills and experience

Giuseppe Falvo D'Urso Labate

The only partner who, in addition to having extensive knowledge in the field of development of the product in question (certified by a PhD program), has business experience, having been the owner of a company.

Diana Massai

With a degree in Nuclear Engineering and a European PhD in Biomedical Engineering from the Politecnico di Torino, in recent years has acquired research experience as testified by the many publications to which she has contributed (26 since 2006).

Umberto Morbiducci

Head of the Cardiovascular System Engineering course at the Politecnico di Torino, has worked as a researcher at the same University and the Department of Health. He is the author of 79 scientific publications since 2000 to date.

The entrepreneurial team has a strong technical connotation which, whilst constituting one of its strengths, also means that it will require other company figures able to bridge the gaps in terms of management and administrative skills. A key role in the initial phase will be that of Dr. Falvo D'Urso Labate who will need to address in more detail the coordination of activities.

The team, in an attempt to maintain the internal structure as small and agile as possible, will initially cover many of the key roles; only from the third year onwards is the entry of skilled personnel foreseen in order to assist them.

Biomicron Srl

Although not directly part of the team, the collaboration of the industrial partner Biomicron Srl will be decisive. The Turin company will provide added value to the venture both in terms of entrepreneurial culture as well as managerial and financial support.

6.6. Market Assessment

Market analysis has focused on the medical sector, in particular tissue engineering, which is part of the multidisciplinary red biotech field (red biotech is the branch of biotechnology dealing with human health through the application of biomedical and pharmaceutical processes to treat diseases).

The target market of Bioexpansys is international. For strategic reasons related to the geographic proximity of potential partners and easier customer accessibility, the market analysis is currently focused on Italy, even though preliminary investigations have begun on European and non-European markets.

Data emerging from scientific articles and reports show that the Italian biotechnology sector is young and in continuous evolution; companies, in fact, were mostly founded between the late '90s and early 2000s. There are high expectations of profound changes associated with the development of "engineering" medical techniques, such as for example the regeneration of damaged tissues and organs starting from the patient's cells, which would replace the current reparatory surgical techniques such as removal (when possible) or transplantation. These changes would also result in more personalized therapies, better quality of life and, certainly not to be underestimated, the possibility of solving the constant shortage of transplant tissue and organs.

6.6.1. Potential customers

The market which the spin-off intends to address is that of organizations.

Currently, in Italy, bioreactors are mostly used in research, with little use in hospitals, mainly due to weak economic and financial support which is why Bioexpansys, entering the domestic market, will in the first instance target the following facilities:

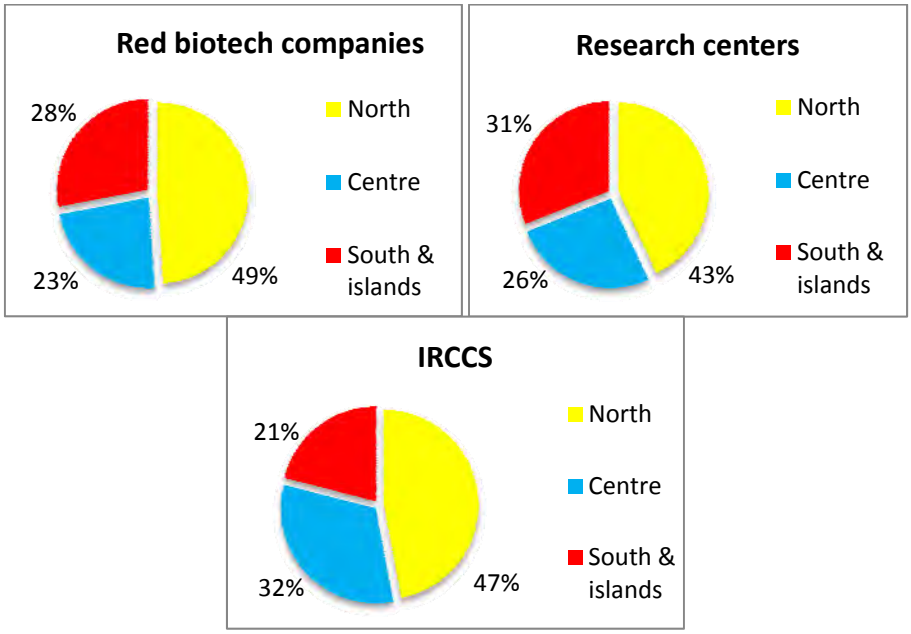
- research centers;
- IRCCS (Scientific Hospitalisation and Treatment Institutes).

The IRCCS are a combination between hospitals and research centers in the true sense of the word since they deal with both research and testing of innovative clinical procedures.

Given the difficulty for a small company to become rapidly known on the market by the most advanced centers, only at a later time will a communication channel be established with:

- red biotech research companies;
- hospitals.

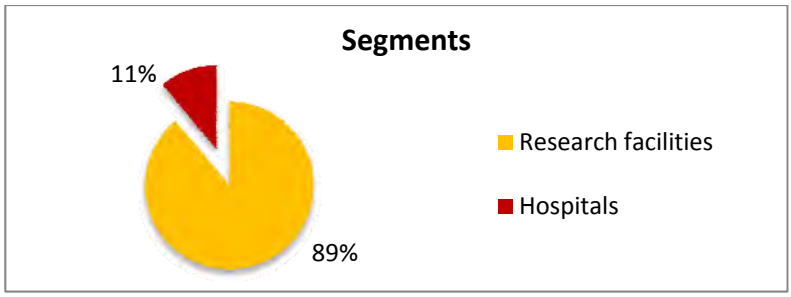
The initial step will be to enter the market in the Central and Northern part of Italy and later to spread across the entire peninsula; the reasons for this strategic decision are shown below in the charts concerning the different concentrations of the institutes mentioned above.



The European market, on the other hand, is more developed than in Italy; bioreactors are currently used for the production of tissue also in hospitals. Given the hypothesised market penetration strategy, activity outside the national borders will not start before at least six years and, therefore, the market analyzed in this document is limited to Italy. Only in the future will these structures be potential customers, with the possibility of placing multiple bioreactors in a single facility.

6.6.2. Market segmentation

From the general list of potential customers, two main segments have been identified, each characterized by particular needs and issues: research facilities and hospitals. Below, the percentage distribution and detailed characteristics distinguishing one segment from another are shown graphically. Although the IRCCS are considered a hybrid, their needs and characteristics concerning the purchase of medical devices liken them more to research facilities.



The percentage values of individual segments were calculated by dividing the number of institutes of the segment in question by the number of centers analyzed (values are shown in Table X below).

Research facilities (red biotech companies, research centers, IRCCS)

Requirements: devices must be capable of producing autologous tissue (such as, for example, cardiovascular and nerve tissue) and carrying out research on dynamic cultures, starting from already differentiated or stem cells.

These customers will be more interested in a more customizable product (multi-tissue and multi-chamber), with disposable components and software for data analysis and monitoring.

Relations: to establish a stable relationship of trust, personalized direct support will be provided (both for usage/maintenance training as well as replacement of disposable items). In addition, to further satisfy the customization requirements of a number of these, the possibility to create or modify product components with a co-creation or co-engineering approach has been hypothesized.

Ability to pay: research facilities constitute the market segment with the greatest need for bioreactors; the problem is certainly their reduced availability of funds and investments required to implement and expand their activities. Within the segment, the IRCCS are the institutions with the most financial resources.

Main communication channels: exhibitions, website, medical congresses, medical journals and direct advertising.

Hospitals

Requirements: devices in this segment must be capable of producing quality autologous tissue in order to meet the needs not satisfied by therapeutic transplants.

These facilities are therefore more interested in a product which is simple to use and is automated, with data monitoring software.

Relations: to establish a stable relationship of trust, personalized direct support will be provided (for usage/maintenance training). Compared to the previous segment, less information and relational exchange is envisaged.

Ability to pay: hospitals have more funds available but, in contrast to research facilities, at present in Italy they do not require a significant number of bioreactors.

Main communication channels: website and medical congresses.

From primary and secondary research, estimates of the number of customers currently using a bioreactor and involved in regenerative medicine and dynamic cell culture have emerged.

The final number is useful to establish the range of users which can be served.

Table 32: Sources: BioInItaly report and Blossom associates report.

	Type of facility	Number	Density %	Investment (million €)
RESEARCH FACILITIES	Red biotech companies for tissue engineering and dynamic cell culture	134	89%	188
	Research centers (*)	300		NA
	IRCCS	40		200
HOSPITALS	Hospitals	56	11%	NA
	Total	**530**		

(*) includes university departments and centers, public and private non-profit research centers and CNR (National Research Center) involved in technological research and development in the biotechnology field.

Secondary research carried out has indicated that the number of bioreactors present in Italy today is around 100, a figure significantly lower than the total number of potential customers just shown (530). For this reason it can be affirmed that the market is not yet saturated and there are significant sales prospects.

To estimate how the Italian market will move in coming years, we relied on the growth rates of the past four years, incorporating the gradually increasing revenue and investment estimates for this area of medical research. The analysis gave rise to the following table:

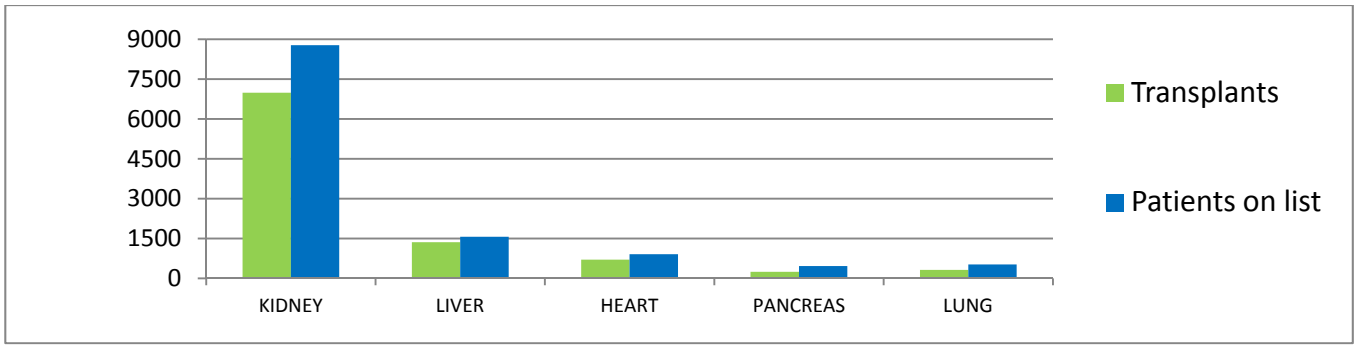
Table 33: Sources: BioInItaly report and Blossom associates report.

Type of facility	Percentage growth	2015
Red biotech companies	+40%	188
Research centers (*)	+3%	309
IRCCS	+3%	42
Hospitals	+2%	57
Total		**596**

(*) includes university departments and centers, public and private non-profit research centers and CNR (National Research Center) involved in technological research and development in the biotechnology field.

Reading the opinions of experts in the field and looking at what more advanced countries than Italy are doing, it is expected that, in a more distant future, the facilities most likely to use tissue engineering products created in vitro will be hospitals which will use them to treat serious problems such as cardiovascular disease, damage from strokes and extensive trauma and cancer-related consequences. This will contribute to an increase in the overall growth of use of bioreactors in the various facilities.

Focusing on the issue of the need to repair/replace organs related to the insurgence of cancer, from the website of the Ministry of Health, the gap last year between the number of people on the waiting list for a transplant and the number of people actually operated was analyzed.



The large gap currently present is immediately clear.

Bioexpansys, with the objective of exploiting the market potential associated with this gap, will initially propose a bioreactor for cardiovascular tissue, as well as that for epithelial and muscular tissue, with a view to offering in the near future devices for the regeneration kidney, liver and pancreas tissue.

6.6.3. Concluding market analysis

The tissue engineering market is still a niche market in Italy; the main activity carried out is research, but with a strong potential for growth over the coming years (suffice it to mention private investment in the *advanced therapies* sector has increased fivefold in the last five years worldwide). On the other hand, the proposed product cannot be considered that new, given that there are already bioreactors with features similar to that designed by Bioexpansys, which is why, following the Ansoff matrix model, one must speak of "Market Development", that is of an existing product with high prospects of diffusion in hitherto unexplored market segments.

6.7. Industry Assessment

Bioexpansys will be positioned in the value chain for the supply of devices for medicine and scientific research alongside companies supplying research centers, biotechnology companies and medical institutions with consumables and equipment which are essential for them to carry out their activities. The specific area in which the company will compete will be that of dynamic cell cultures, which includes manufacturers and distributors of consumables and equipment necessary for the culture (scaffolds, cells, culture liquids and bioreactors/incubators).

6.7.1. Industry characteristics

Intellectual property is of prime importance in the industry since we are dealing with advanced technologies. The possibility of protecting one's innovations in an effective manner hinges on patenting and, reversing the perspective, patenting of certain technologies by competitors could pose significant barriers to research and development.

Finally, the company must pay attention to how it interacts with customers since this can heavily influence the relationships of rivalry with the competition. The sale of a bioreactor, bearing in mind that many customers will be public bodies and, since the volumes purchased by the individual customer will not be high, could be a very structured and complex transaction in terms of information and very infrequent over time.

6.7.2. Competitors

Considerations concerning the nature of present and future competition lead to uncertain conclusions. Of the 19 competitors in the sector, only 4 are considered direct and therefore particularly dangerous; these are: Bose ElectroForce, TGT, Bio Tools and ZellWerk.

The costs of entering the market for the production of devices such as the one to be developed are relatively low, if one excludes research and development costs, and do not represent an insurmountable obstacle to entry for those in possession of specific scientific know-how. The medium-low entry barriers, on the one hand, are conducive to a high level of competition while, on the other, facilitate Bioexpansys' entry. It should, moreover, be noted that the company will face competition from both globalized companies as well as small businesses similar to its own, although it is expected that competition will be on various levels and degrees of intensity.

As a substitute for bioreactor technology are traditional cell culture techniques such as Petri disks, although these only represent a viable alternative for certain types of use, given that bioreactors are a direct technological evolution of the latter.

6.7.3. Concluding industry analysis

The target industry analysis can be concluded by highlighting and comparing the following key aspects:

- internal rivalry: average;
- barriers to entry: medium-low;
- supplier power: low;
- customer power: average;
- substitute products: medium-low.

In conclusion, we can say that the characteristics of competitors and alternative products pose certain threats to the possibility of success but there are still ample opportunities for a satisfactory evolution, constituted mainly by the absence of a dominant product design, of standards to be complied with and by the limited number of truly established competitors (in “ANNEX VI - Industry assessment” a complete report of the analysis carried out can be found).

6.8. Market and Product Strategy

The strategic phases, cornerstones for the implementation and success of an enterprise, can be summarized according to the following time line:

- prototyping phase (from month 1 to 18): initial phase comprising the creation of the first patented and certified Bioexpansys prototypes, ready to be tested by external personnel;
- industrialization phase (from month 19 to 26): intermediate phase in which the production and commercialization phases of the products under development are designed;
- production phase (from month 27 onwards): last phase in which finished products, ready to be sold to contacted customers, are produced.

For further information on these phases and details of activities, see ANNEX VII and VIII.

6.8.1. Market entry strategy

In line with the fact that there will be no production in the first two years, the market entry steps will be:

Year 3 → Central and Northern Italy: Bioexpansys will begin to promote the devices in areas close to the headquarters due to both the geographic proximity (as well as lower promotion and transportation costs) and the increased density of centers and companies involved;

Years 4-5 → coverage of the entire domestic territory: the fundamental goal is to cover the entire country, reaching even those facilities which are currently not aware of the product or do not have sufficient funds to invest in it;

Year 6 and beyond → entry into the European market: Bioexpansys will attempt to expand beyond the national borders, reaching the United Kingdom first. The choice is not random since the entrepreneurs have already initiated cooperation with Imperial College London which will be important for the possible expansion of sales.

The decision to focus on the Italian market in the initial product commercialization phase is related primarily to the scarcity of products on the market (only a few research groups in Italy are working on the development of these devices and are able to offer a competitive product, thus diverting those working in this field toward foreign products). Moreover, geographic proximity will facilitate effective management of the initial development phase in which end-user feedback will be of fundamental importance). Only at a later stage will the product be in a position to face the heavier foreign competition.

6.8.2. Entry timing and future threats

The timing of market entry is one of the most important success factors for a company and that is why Bioexpansys proposes to enter the market "rapidly" (bearing in mind the product in question) for the following reasons:

- get customers used to the product offered at the earliest;
- the product developed by the company has technological innovation as its main strength;
- the market is growing, with new startups and spin-offs appearing every year; Bioexpansys must avoid being "outclassed" by new competitors with brand new technologies.

Obviously, a good timing analysis must take into account possible future threats to be faced. The most likely threat in the short term will be linked to the entry of new companies similar to Bioexpansys, in which case the objective will be to leverage on its ability to innovate and on product awareness. Intensification of competition from larger competitors on the market is possible in the long term when such companies could decide to exploit their brands and increased availability of resources. A second potential threat is represented by the dependence on Biomicron Srl, sole supplier of ad hoc product components for the company's bioreactor; this potential problem is mitigated, however, by the participation of Biomicron Srl in the startup as a shareholder.

6.8.3. Innovation strategy

The innovation that Bioexpansys intends to offer the market is mainly in the area of product innovation. The company plans to offer a product capable of bringing technical improvements via:

- new tissue stimulation features with the capability to operate at frequencies and amplitudes currently not available on the market;
- use of new materials and disposable components.

In addition, a policy aimed at forging continuous and collaborative relations with customers will be pursued, in contrast with that currently implemented by direct competitors, via:

- advanced technological consulting for development and upgrades;
- telephone and remote support;
- collection and trade-in of used products.

These channels facilitate Bioexpansys in combating stronger competitors since they do not offer these services (or do not offer them simultaneously).

6.8.4. Sustainability and inimitability over time

The factors sustaining the product on the market over time are:

- patent;
- lower sales price compared to the average competition price;
- rapid innovation over time thanks to a rapid economy;
- simultaneous provision of additional services.

Sustainability of the competitive advantage is closely related to the degree to which it can be imitated by competitors. Regarding the use of materials (not in themselves innovative but innovative inasmuch as not yet used for these applications), imitability appears to be simple enough whilst it would certainly be more difficult to imitate the mechanical tissue stimulation management features since these are the result of the consolidated experience of the entrepreneurs in this sector. In particular, for greater protection, it is expected to be able to patent the grip technology (i.e. the devices which keep the tissue in place in the culture chamber), the algorithm which manages the internal cultivation process and the microscopic analysis methods/interfaces.

6.8.5. Strategic resources and core business

The strategic resources the new company considers to have and which it intends to use to support the startup and subsequent development are, in addition of course to a valid product, the know-how of the entrepreneurial team and the partnership with Biomicron Srl. Lower intensity, but not less important, collaborations which the researchers have begun to establish in recent months have been with the University of Turin, the Politecnico di Torino and Imperial College London.

The experience gained in several research projects in the specific field of bioreactors for regenerative medicine should provide the right initial impetus for the company in this nascent market, allowing Bioexpansys to significantly reduce the time and effort necessary to create the first prototypes and thus reducing research and development costs related to the first products to be developed. The partnership established with Biomicron Srl should also ensure the manufacturing and technological solidity in order to move from the product development to the product industrialization phase, as well as an important transfer of know-how with regard to the dynamics of administrative, financial, production and commercial management.

The core activities, which should constitute the hallmark and possible source of competitive advantage, on which the company will focus are:

- product design and industrialization (R&D);
- assembly;
- sales and marketing;
- after-sales support and maintenance.

In particular, the first will be the pivot around which the chances of success of the venture revolve, since efficient design can reduce time-to-market and provide a product with satisfactory performance. Obviously the activities listed above should be adequately supported by all the other activities necessary to provide the finished product (marketing, quality control, inbound/outbound logistics and company management).

6.8.6. SWOT analysis

Below is the summary table of the SWOT analysis carried out; the explanations of the individual items can be found in “ANNEX X - SWOT Analysis”.

SWOT ANALYSIS	**OPPORTUNITIES** No dominant design in the sector Proximity with academia Few affirmed competitors Constant and collaborative customer relationship	**THREATS** Competitors and university spin-offs Alternative, substitute patentable technologies Cost increase risk Key resources difficult to find Dependence on Biomicron Srl for ad hoc components
STRENGTHS Entrepreneurs' know-how Patentability Technological innovation Internal assembly Comprehensive service offer and niche product Production capacity and sales network ensured by Biomicron Srl. Competitive quality/price ratio	Commercial partnership with Biomicron Srl to exploit market growth potential Emphasize communication on quality/price ratio (science exhibitions, specialized press, etc.)	Use patents Exploit the partnership with Biomicron Srl to share costs and investments Control product distribution so as to reduce the risk of imitation Biomicron Srl as a shareholder
WEAKNESSES Limited entrepreneurial know-how Long time-to-market High initial investment and financial weakness	Collaborations with academia Obtain a large pool of loyal and collaborative customers	Reduce time-to-market to address competing alternatives Focus on external collaborations to tap into additional resources and know-how

6.9. Marketing and Sales Plan

The Bioexpansys philosophy, rather than focusing on maximizing profits through technological superiority, will be related to finding a profitable satisfaction of needs, with a strategy capable of bridging the gap between market requirements and the current technological offering.

6.9.1. Product

The company, at least initially, will align itself with that offered by competitors, selling products, spare parts, disposable components and maintenance and support services independently one from another, like any other manufacturer. Simultaneously with the sale, it will also provide a limited period warranty (2 years, as the main competitors) and sufficient disposable components to carry out 2-5 cell cultures.

The product itself, however, will not be the only revenue stream, since the company will also offer consulting services to develop customizations and, via agreements with the partner, will provide the necessary disposable elements to use the bioreactor. In particular, these components will be produced ad hoc by Biomicron Srl and will only be compatible with the bioreactor of the spin-off.

6.9.2. Pricing structure

In terms of pricing, the company is oriented towards a traditional cost mark-up mechanism, despite this often being inadvisable for a startup which typically has high production costs for the first product samples put on the market. Bioexpansys believes, however, that in its particular business case this will not be an obstacle, primarily because it is entering a market in which, at the moment, it is certainly not the economies of scale which make the difference and, secondly, because, also on the basis of the experience of the entrepreneurial team, it will direct design activities from the outset towards production cost containment.

The price of 55,000 € currently established by Bioexpansys for the basic model was estimated as a trade-off between the prices of (dynamic) bioreactors currently on the market and the entrepreneurs' assumptions concerning production costs.

The disposable culture kits will be produced and sold by the partner Biomicron Srl to distributors at a price of 10 €. On such price, Bioexpansys, through contractual clauses, will earn a royalty of 20%, ensuring an equitable distribution of margins between the two companies.

Finally, the consulting service, particularly significant in the early years of the life of the company, will be offered at discriminatory prices. In particular, if consulting is requested for minimum upgrading of a standard bioreactor, the price of this service will be competitively priced at cost (approx. 80 €/h). Should, on the other hand, the customer request the ad-hoc development of a bioreactor, such design service will be offered at a price of 160 €/h, which reflects the current market rates.

6.9.3. Promotional activities

Given the technological nature and specificity of the product, promotion must be designed in a precise and accurate manner. First of all, promotional activities can be divided into two main areas: advertising and convincing the individual customer to buy. It is clear that the activities in the second category will be implemented when there are potential customers interested in purchasing the product. This is why the company will start activities in different phases of development, as shown in the following table: activities able to generate greater visibility will already start in the final prototyping phases, while other very customer-specific phases will be undertaken at the end of the industrialization period and more massively in the “production” phase.

End of prototyping/Industrialization	Production
Website **Scientific articles** **Exhibitions:** **("Termis" and "World Stem Cell and Regenerative Medicine World Congress")** **PR** **Demonstrations**	Advertising in specialized press Free test culture Organization of training days Warranty, discounts on repurchase and trade-ins

It is estimated that the budget necessary to carry out the activities listed above for the first three years is approx. 50,000 €, maintaining a certain amount of flexibility according to the funding and opportunities provided by the industry sector.

In the short term it will be the company itself which will take its product to the customer to stimulate purchase, mainly due to direct contact by distributors and acquaintances of the entrepreneurs. In the long term, on the other hand, the goal will be to move to a new strategy, in which it will be the customers themselves who will request the product to the distribution channels or directly to the company.

The spin-off could then adopt a simple scoring model to evaluate which activities are more effective at any specific time, in order to more efficiently allocate the resources available (as initial data the evaluations contained in "ANNEX XI - Marketing and sales plan" can be used.

6.9.4. Distribution

The company, for the initial sales, will rely on the one hand on the distributors with which the partner Biomicron Srl already cooperates and, on the other, on the direct knowledge of researchers involved in development and on company product specialists, given that this is an expensive, complex-featured product with limited sales volumes. Moreover, purchase requires a two-way communication, even more so since this is a bioreactor developed by a startup which has not yet established itself in the sector. Contact with the customer is very important to resolve any doubts and for reassurance, thus increasing the likelihood of purchase. In particular, the figure of the distributor must on the one hand "promote" the bioreactor towards customers, put them in contact with the spin-off and offer the sale of disposables; the distributor will therefore have a vested interest in finding potential customers for Bioexpansys in order to increase the potential future profits from the sale of disposables. In addition, the spin-off will, therefore, represent the actual technical contact for the bioreactor and high value-added consulting services. Product specialists, with technical knowledge, will, on the other hand, take care of customer negotiations and interaction in a broader sense.

6.10. Operations

6.10.1. Production Cycle

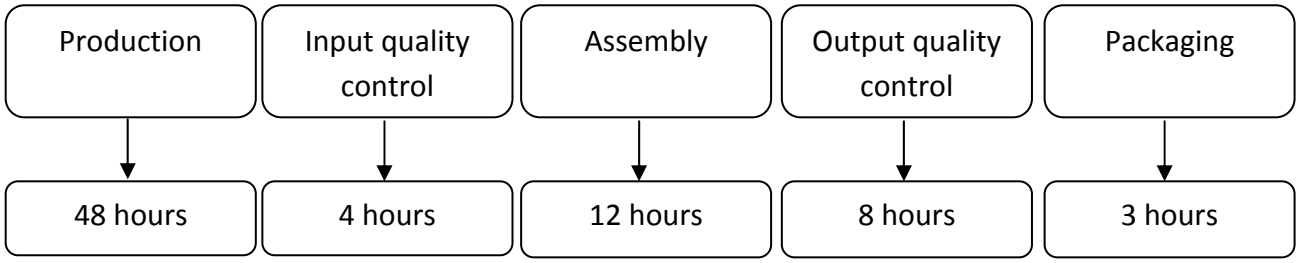
To understand the Bioexpansys production cycle, the individual activities from initial input to customer delivery of the finished product must be analyzed. Below, cycle costs and timings, calculated for a single product, are shown. In "ANNEX XII - Production cycle" individual phases are described in more detail.

Timescale perspective

The core activity of the production cycle is related to product Research and Development which will be predominant, for Bioexpansys, starting from the third year of activity. For this reason and also in relation to projected sales volumes, a PhD student will be hired (1,250 € per month) to support R&D activities. Other

costs regard product certification (5,000 € submission + 1,000 € a year fees) and the patent (10,000 € a year for the first 4 years).

The other activities instead concern:



Total expected time is 75 hours; excluding production, outsourced by Bioexpansys, approx. 27 hours or 3½ working days are required. Taking into account a margin of safety, 4 days have been assumed and this means that each operator will be able to assemble and control a maximum of 5 bioreactors per month. In the immediate future, no assemblers need to be hired.

Customers will be ensured product delivery within 14 working days from receipt of order, corresponding to 18-20 calendar days (for further details see table in "ANNEX XIII - ORDER PROCESS").

These timings have been conceived for "standard" production to the customer, as Bioexpansys will do initially; should the customer request a product upgrade, to these timings must be added the design time and associated delays in activities downstream of the production process.

Economic perspective

The following table shows the costs of activities described above.

Activity	Resources	Cost
Input supply quality control + Finished product quality control + Assembly	Equipment (Jigs - Manual gauges - Lasers - Software)	4,000 Euro (1st year) 1,000 Euro (2nd year) 2,000 Euro (subsequent years)
Packaging	Packaging materials	50 Euro/bioreactor
Input supply quality control + Finished product quality control + Assembly + Packaging	Personnel (apprentice from 3rd year)	15,600€/Year

For details of infrastructure costs, see "ANNEX XIV – INFRASTRUCTURE".

Shipping costs

On the component supplier side, incoming shipping costs are borne by Bioexpansys; on the customer side they will be borne by the customer.

Should the product need to be withdrawn by Bioexpansys due to malfunction, damage not related to misuse by the customer or manufacturing defects, collection costs will be borne by Bioexpansys, as too will be the subsequent re-shipping costs. In this case, however, insurance cover is planned.

Warranty and after-sales service costs

Since sales volumes will be numerically low, even when up and running, and the product should be highly reliable, it is expected that warranty will not constitute an excessive cost for the company. Specifically, a 2-

4% defect rate (decreasing over the years) is expected with the need to repair or replace approx. 3-5 products during the time period analyzed.

Repairs will be carried out by the assembly workers who will diagnose and replace damaged components.

As for after-sales service, it should be noted that the volume of work necessary will be directly related to sales, given that the basic service offering will consist of installation of the device and user training on operation of the same. This will be an activity performed at the customer site which is estimated to take approximately one working day.

In addition to these routine tasks, there will also be the possibility to customize the product to obtain the configuration that best suits the needs of each specific customer. It is estimated that, on average, 10 man/hours of work will be required for a basic configuration procedure (planned for all products sold) and 80 man/hours for more complex modifications involving a re-engineering process for certain product components (it is assumed that this second type of activity will be carried out on 10% of bioreactors sold).

Payment management

Regarding payment of suppliers, timing will be different depending on the supplier, namely:

- external suppliers: payment 60 days from delivery of material;
- Biomicron Srl: payment 90 days from delivery of material.

On the receivables side, customers will be granted 90-120 days payment terms from delivery of the bioreactor; for technical consulting services, on the other hand, an initial fee (20-30% of the total price) will be paid before starting work with the balance at the end of the project.

Geographic perspective

Bioexpansys, a university spin-off, will enjoy the collaboration of the Politecnico di Torino for the development of research in the biomedical field, consulting from the I3P incubator for the first three years of business life and the possibility to use university premises; this will allow the company not to have to immediately support sunk costs that could weigh heavily on initially limited financial availability.

Bioexpansys, since it will assemble parts which the partner Biomicron Srl will produce, will not need to have large plant or machinery dedicated to processing raw materials. For the first three years following foundation of the company, the registered offices will be at I3P, where it will have an office of approx. 20 square meters with rent increasing over time. Moreover, in agreement with the Politecnico di Torino, premises will be available c/o the department to carry out activities which, for reasons of space and technical equipment, cannot be carried out at I3P. Starting from the fourth year, Bioexpansys intends to establish itself c/o Biomicron Srl which will rent space for a total surface area of approx. 70 square meters; in this way it will not only benefit from closer contact with its partner but also from lower rental costs.

In “ANNEX XV – LOCALISATION” individual costs are shown in detail.

Warehouse

Production will mainly take place to order and, therefore, there will be no finished goods warehouse. There will, however, be a warehouse, located in Biomicron Srl, to store individual components (actuators, pumps, etc.), dimensioned according to procurement lead time (6 weeks between order and receipt of product) cost of components and quantities of product sold.

6.10.2. Sales

To estimate sales volumes, two main scenarios were analyzed in which, in the first case, the number of red biotech companies as potential customers was taken into account and, in the other, was omitted. The reason for this decision is the uncertainty that a small nascent company will be able to get itself known and sell its product to private biotech companies in the years immediately following its entry on the market.

The entrepreneurs, once on the market, have set themselves the sales target to install the product in 10-20% of research facilities. Bearing in mind the high number of individual centers presented in the chapter "Market Assessment", the following are two possible sales scenarios (best and worst case).

Best case: scenario with target of 20% of the entire segment

88 bioreactors in research facilities.

	Year 1	Year 2 (*)	Year 3	Year 4	Year 5
Sales	-	3	10	25	50

(*) this is the year prior to entering the market; according to agreements, 3 prototypes are sold

Worst case: scenario with target of 10% without red biotech companies

	Year 1	Year 2 (*)	Year 3	Year 4	Year 5
Sales	-	3	3	10	20

36 bioreactors in research facilities.

(*) this is the year prior to entering the market; according to agreements, 3 prototypes are sold

In both scenarios, technical consulting starts at the end of the first year for the development of prototypes, increasing over time due to the expected sales growth.

The commercial partner, together with the experience of the entrepreneurs and the technically advanced product, justifies the possibility of also selling to red biotech companies, as foreseen in the "best case" scenario.

6.10.3. Regulation

Regarding the possible regulations to which the bioreactor might be subject, the case in which sales are made to research facilities or clinical facilities must be distinguished.

For the first segment, the reference standard is 93/42/EC Medical Devices, which refers for more specific cases to standard 13485, but both present no major regulatory obstacles for the product.

Should the device be addressed to clinical facilities things are somewhat different since the critical issues related to products and medicines used in tissue engineering are not the same.

To minimize if not eliminate these obstacles, Assobiotec and Farmindustria, two important associations for biotechnology development, have put forward a number of practical proposals for discussion to improve the situation; these are:

- proposals for improvement of the authorization process for clinical trials;
- exclusions from the registration procedure (Article 28);
- use of drugs even after the end of the transition period;
- requisites for export to Italy.

In “ANNEX XVII – Regulation” a more detailed discussion of this topic can be found.

6.11. Economics and Financials

The Bioexpansys economic and financial plan has been developed over five years in which the company has analyzed in detail the costs to be borne in creating the company and, above all, in its development, as well as the corresponding revenues related to future sales forecasts based on a worst and a best case scenario. The analysis has been carried out every two months for the first two years of the company and subsequently the analysis was performed every six months. The main key success factors emerging are:

- partnership with Biomicron Srl: key for company development as it is the main supplier and manufacturer of bioreactor components, but also one of the main investors;
- Politecnico di Torino: will provide the university spin-off with research facilities at a commercial price; this will allow Bioexpansys not to immediately have to bear sunk costs.

The milestones which Bioexpansys has set itself in its development plan are as follows:

- Year 1: foundation and startup of activities;
- Year 2: development and sale of the first prototypes;
- Year 3: entry on the market;
- Year 4: exit from the Incubator and rental of premises in Biomicron Srl;
- Year 5: substantial increase in revenues through a strong growth in sales and consulting activities.

The economic-financial plan is based on company assumptions, coming from primary and secondary research carried out and described in previous sections.

Subsequently, the key economic and financial aspects will be analyzed; for further details please refer to “ANNEX XVIII - Economics and Financials”.

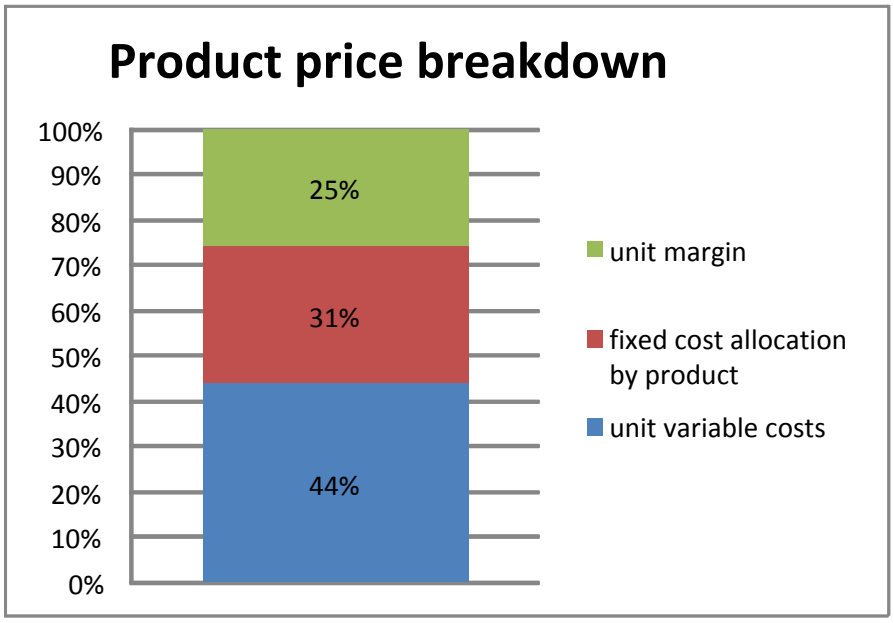
6.11.1. Sales volumes and price

Sales volumes have been forecast based on two scenarios:

TIME	YEAR 1		YEAR 2		YEAR 3		YEAR 4		YEAR 5	
Scenario	**1st half**	**2nd half**	**1st half**	**2nd half**	**1st half**	**2nd half**	**1st half**	**2nd half**	**1st half**	**2nd half**
Best Case	0	0	1	2	4	6	11	14	24	26
Worst Case	0	0	1	2	1	2	4	6	8	12

As the table shows, in the first year the company will not enter the market since the product is still under development; in the second year the company will sell the first prototypes which will also act as testers to improve the product and from the third year onwards it will enter the market with the first customer sales. Bioexpansys expects substantial growth in sales by the fifth year onwards since the industry is in full development and, moreover, over the years the company will seek to establish its brand on the market. Sales volumes also include consulting for product development and upgrades; of course, consulting will increase with the increase in bioreactor sales and represent an additional source of profit.

The basic bioreactor sales price is 55,000 € and is composed as follows, in the “worst case” scenario:



In the second year, however, prototypes will be sold at 40,000 € since the customer buying the bioreactor will be used by Bioexpansys as a tester for product development.

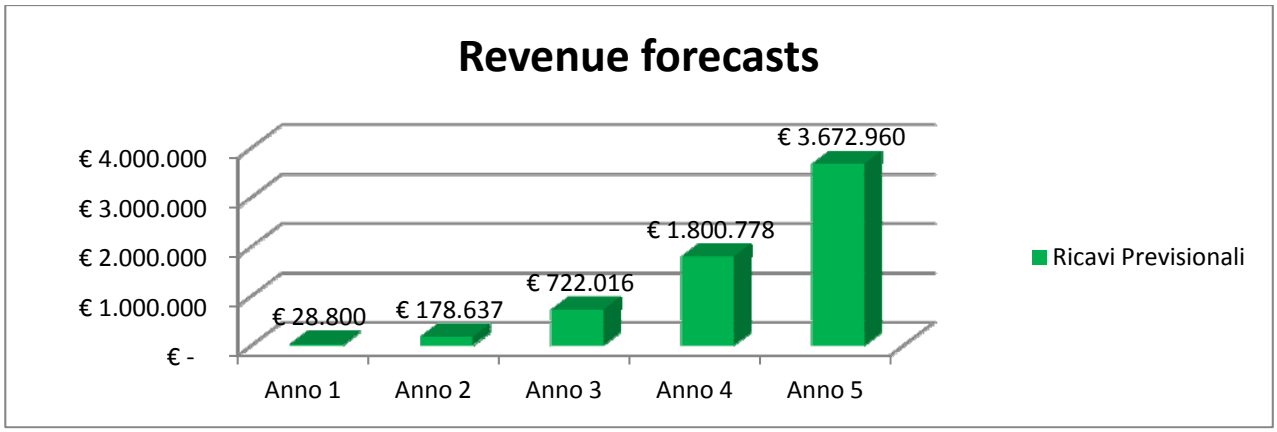
6.11.2. Income Statement items

The table below shows the forecast income statement for the "best case" scenario. Below, a deeper analysis of the constituent elements will be made.

	Year 1	Year 2	Year 3	Year 4	Year 5
SALES	€ 28.800	€ 178.637	€ 722.016	€ 1.800.778	€ 3.672.960
COST OF SALES	€ 10.800	€ 79.290	€ 290.956	€ 695.084	€ 1.555.284
	€ 18.000	**€ 99.347**	**€ 431.060**	**€ 1.105.693**	**€ 2.117.676**
OVERHEAD	**€ 43.671**	**€ 91.504**	**€ 293.340**	**€ 417.243**	**€ 497.476**
Research & development	€ 19.351	€ 54.044	€ 93.440	€ 104.080	€ 102.413
Sales and Marketing	€ 2.800	€ 19.800	€ 50.200	€ 131.200	€ 201.200
General Administration & Finance	€ 21.520	€ 16.760	€ 127.300	€ 159.563	€ 160.763
Cost of labor for production and consulting	€ -	€ -	€ 20.400	€ 20.400	€ 30.600
Cost of maintenance of patents and certifications	€ -	€ 900	€ 2.000	€ 2.000	€ 2.500
	-€ 25.671	**€ 7.842**	**€ 137.720**	**€ 688.451**	**€ 1.620.200**
DEPR. & AMMORT.	**4800**	**5175**	**4300**	**3475**	**3683**
	-€ 30.471	**€ 2.667**	**€ 133.420**	**€ 684.976**	**€ 1.616.517**
INTERESTS	**€ -**	**€ -**	**€ -**	**€ -**	**-**

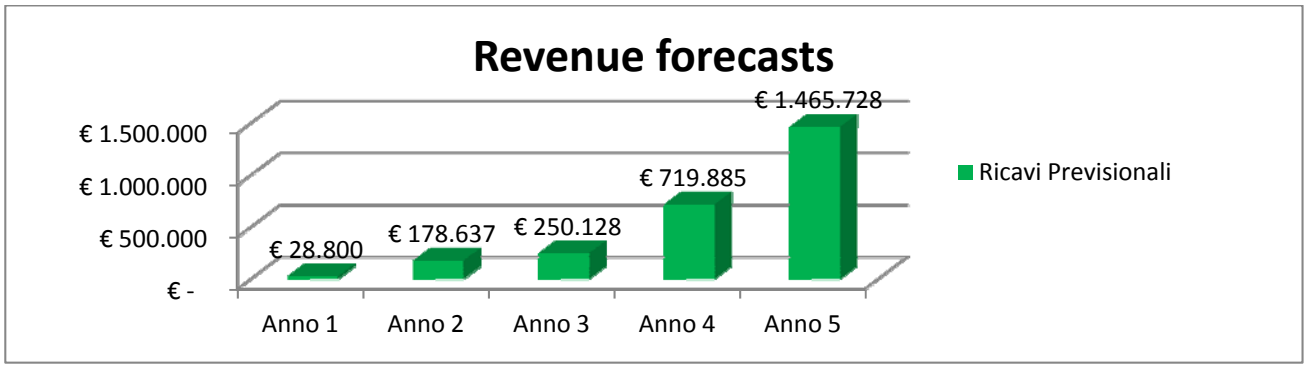
Revenue forecasts

Bioexpansys has taken into consideration in its forecast the sale of a single bioreactor model, postponing expansion of the product range to a more stable later stage, outside the time horizon of this document. Revenue forecasts also include consulting which Bioexpansys will provide to sell its products. In the "best case", the chart showing the trend of expected company revenues is the following:



In the first two years, to finalize and partially self-finance development, only technical consulting services will be provided and a number of prototypes implemented (2nd year). From entry on the market in the third year, forecast revenues based on sales targets more than double every year. As previously mentioned, revenues have been estimated considering the product basic sales price, consulting and royalties on disposables; the latter two sources of revenue, however, represent a reduced variable amount (on average 10%). The estimated values increase from approx. 700,000 € in the third year to more than € 3.5 million in the fifth year, demonstrating the ample margins of expected growth in the sector.

Moreover, the expected revenue trend in the "worst case" is also significant. In this case, the forecast values grow from approx. 250,000 € in the third year to more than € 1.4 million in fifth.



Variable costs

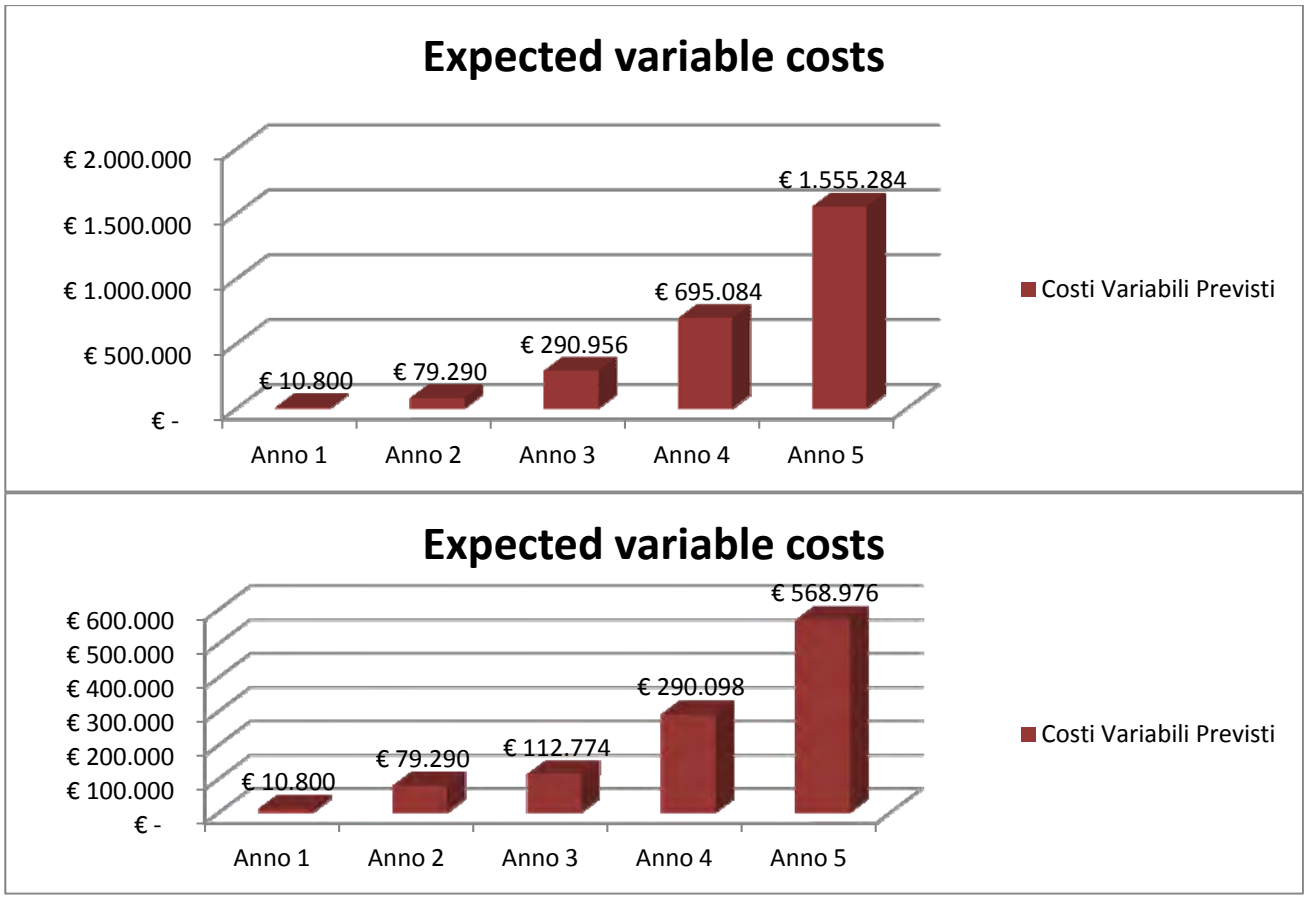
The expected variable costs can be divided into three categories:

variable product costs: include component costs for bioreactor construction and commissions for agents and distributors;

variable consulting costs: represent the hourly rates charged for consulting;

variable warranty costs: represent those costs incurred by the company in case of malfunction of the product under warranty (the average probability of a product fault is assumed to decline over the years due to the experience acquired).

The charts show variable cost trends in the "best case" and in the "worst case".



The variable cost trend is increasing since it is tied to sales. There are no planned economies of scale or scope with regard to assembly and outsourced component manufacture, unlike what could be expected with regard to research, marketing and other overhead costs covered in the next paragraph.

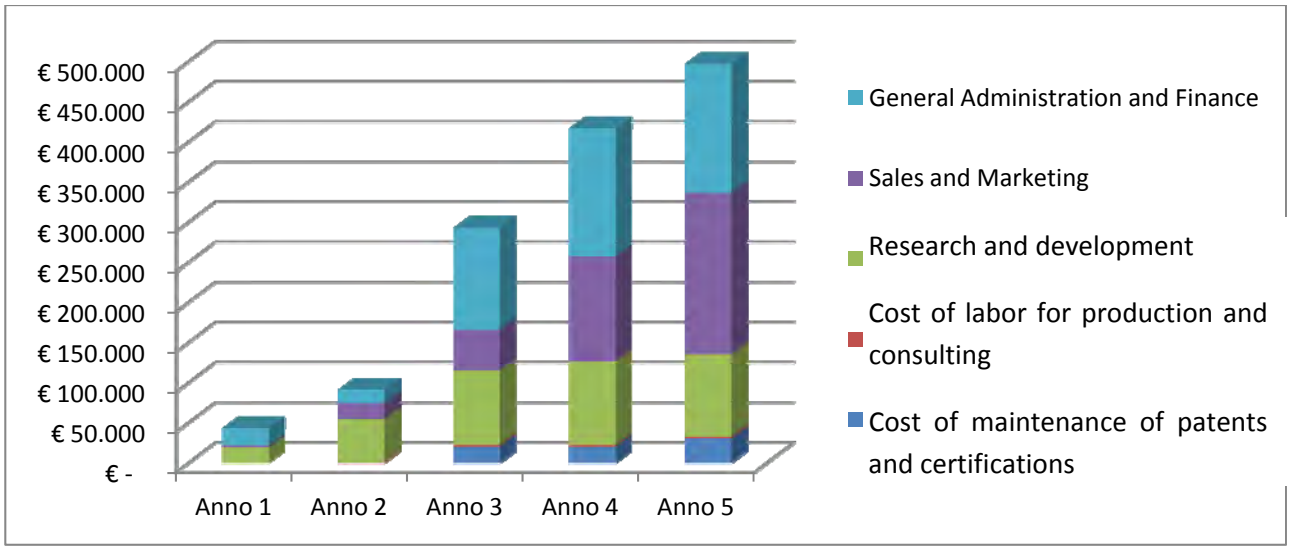
Overheads

This item includes the following categories:

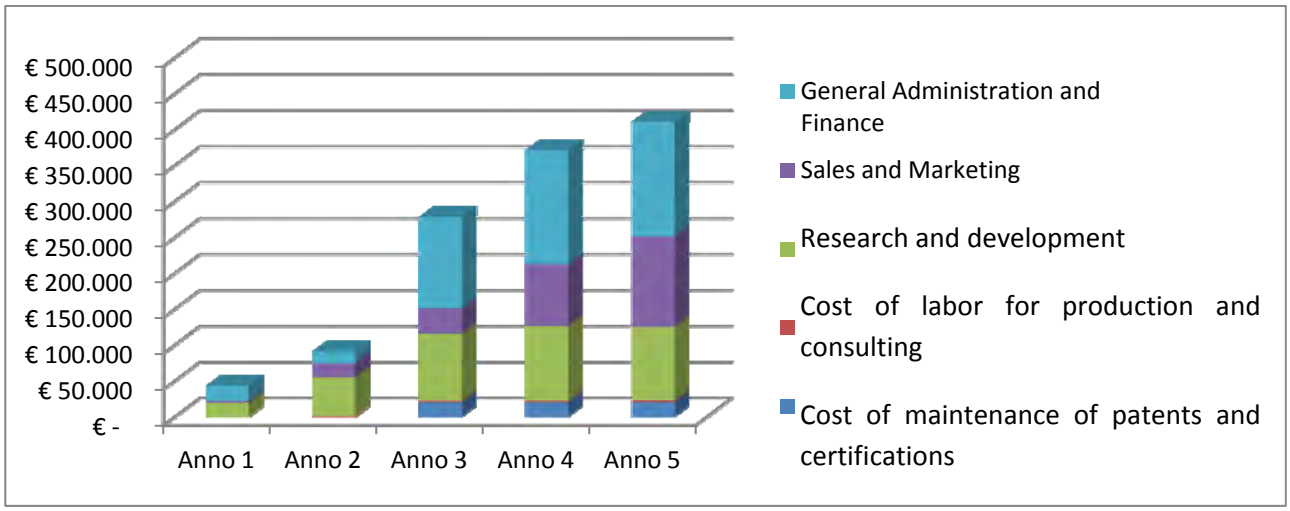
- labour cost for production and consulting;
- patent and certification cost;
- research and development (labour, materials, services);
- Sales & Marketing (marketing and personnel expenses);
- General Administration and Finance (personnel, outside consultants, utilities, premises rental).

The charts below show that over the years the company will increase investments in R&D, marketing and customer relations and in skilled professionals in order for the company to grow and develop. R&D investments are essential for product development and creation of value for the future, while marketing investments will enable the company to establish itself on the market, affirm its brand and acquire the trust of its customers through sales personnel (“product specialists”).

In the “best case” overheads are as follows

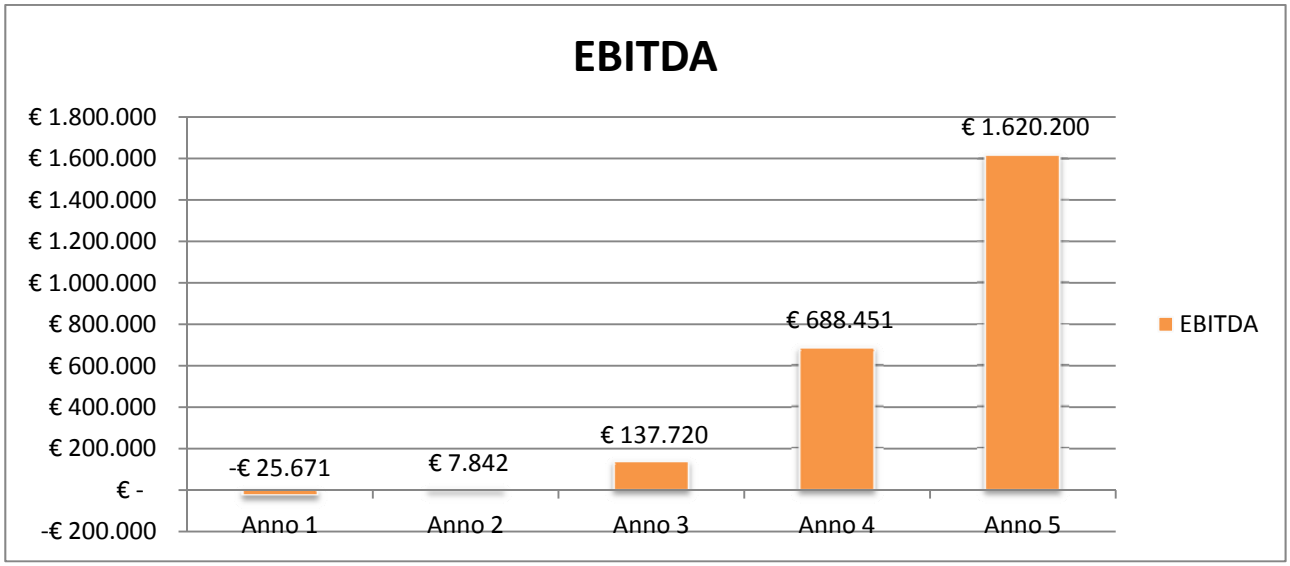


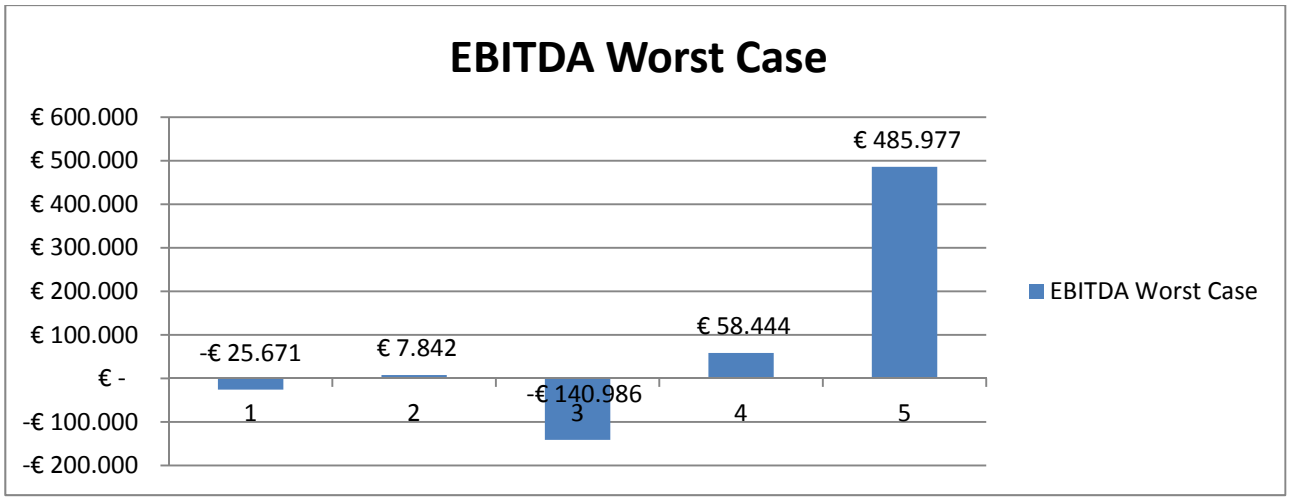
and in the "worst case" as in the chart



EBITDA

The following charts show the EBITDA trend over time for the "best case" and "worst case", respectively.



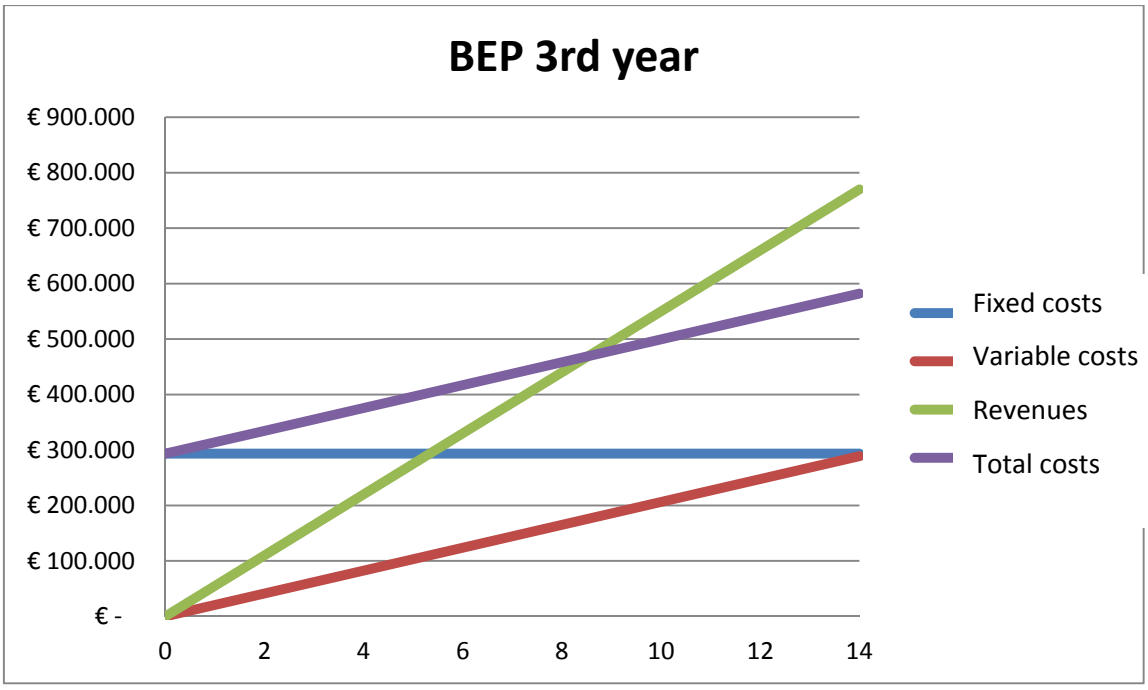


The EBITDA chart shows how the company already manages to cover operating costs from the second year onwards ("best case" scenario), mainly through technical consulting for development of the first bioreactors (prototypes). In subsequent years, with market entry explosive growth is expected so as to reach approx. 1.6 million € in the fifth year, i.e. approx. 44% of revenues. The high margins generated by the company are to be attributed to the advanced technological knowledge of these devices and the research behind them. Most of the cash proceeds are in fact reinvested in order to cover the high product and know-how investments, as will be analyzed in the section concerning cash flow.

In comparing the best and worst cases, it should be noted how the margins generated undergo a sharp decline, bringing margins to approx. 490,000 € in year 5 (approx. 31% of the "best case" value). This fact is generated by the presence of fixed research and order management costs of a magnitude comparable to variable costs for this size of company.

Break Even Point

The break-even analysis performed on the first year of launch of the final product on the market shows that the sale of a minimum of 6 bioreactors (equivalent units) would be required for the company to cover all costs. This results in a slight operating loss in the 3rd year in the worst case scenario, since sales of only 5 units are expected, which would result in requiring additional external funding also to cover costs.



6.11.3. Balance Sheet items

The table below shows the forecast balance sheet for the "best case" scenario. Below, a deeper analysis of the constituent elements will be made.

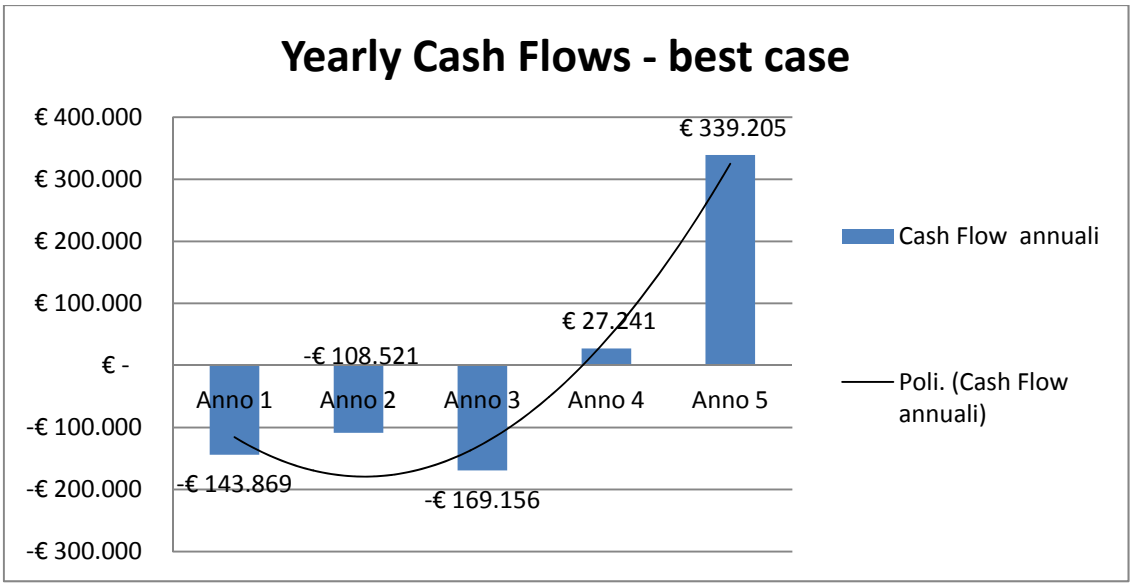
	Year 1	Year 2	Year 3	Year 4	Year 5
CURRENT ASSETS	€ 37.356	€ 127.396	€ 429.464	€ 996.382	€ 2.282.600
LONG TERM ASSETS	€ 90.129	€ 135.989	€ 144.329	€ 146.654	€ 137.638
TOTAL ASSETS	**€ 127.485**	**€ 263.385**	**€ 573.793**	**€ 1.143.036**	**€ 2.420.238**
CURRENT LIABILITIES	€ -	€ 35.310	€ 70.620	€ 160.380	€ 306.020
LONG TERM LIABILITIES	€ -	€ -	€ -	€ -	€ -
SHAREHOLDER EQUITY	€ 127.485	€ 228.075	€ 503.173	€ 982.656	€ 2.114.218
TOTAL LIABILITIES & SH EQUITY	**€ 127.485**	**€ 263.385**	**€ 573.793**	**€ 1.143.036**	**€ 2.420.238**

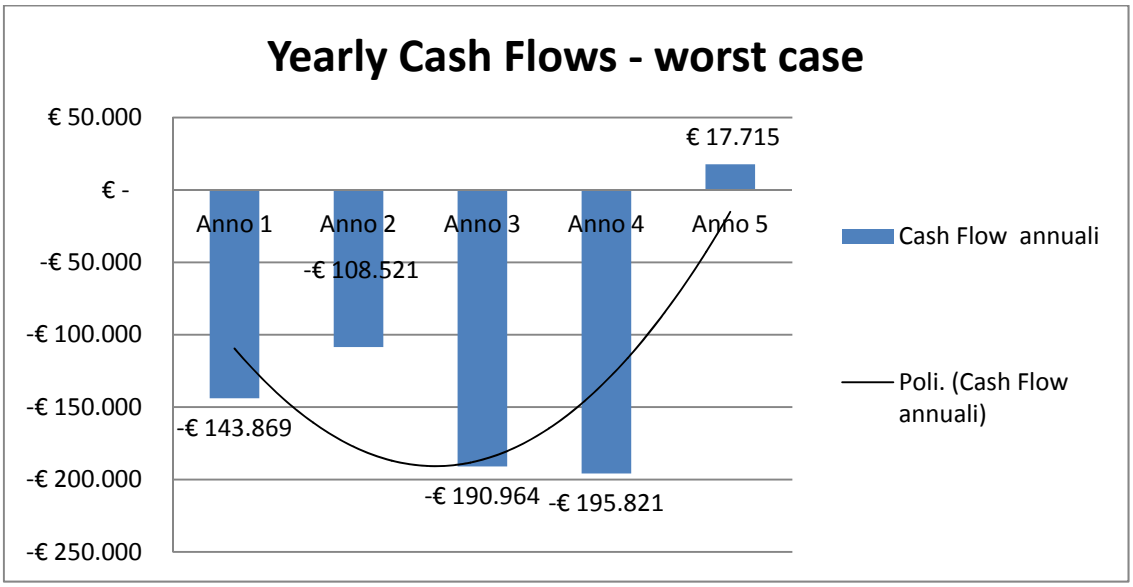
An analysis of the assets of the forecast balance sheet shows a normal increase in value over the 5 years. A substantial imbalance between current and fixed assets, from the third year onwards, can be noted, mainly due to the valuation of patents at the cost of procedures and the know-how of the entrepreneurs not being accounted, as well as the strategic decision to outsource component production. This imbalance is conservative and natural in a technology startup in which outsourcing represents an opportunity to initially reduce fixed assets.

Regarding financing, the all-equity approach is dictated by the difficulty for a startup to obtain bank loans. The item "current liabilities" consists primarily of trade payables whose amount is very small compared to trade receivables. The cause is to be attributed to the difference in timing of payments between suppliers and customers.

6.11.4. Cash Flows

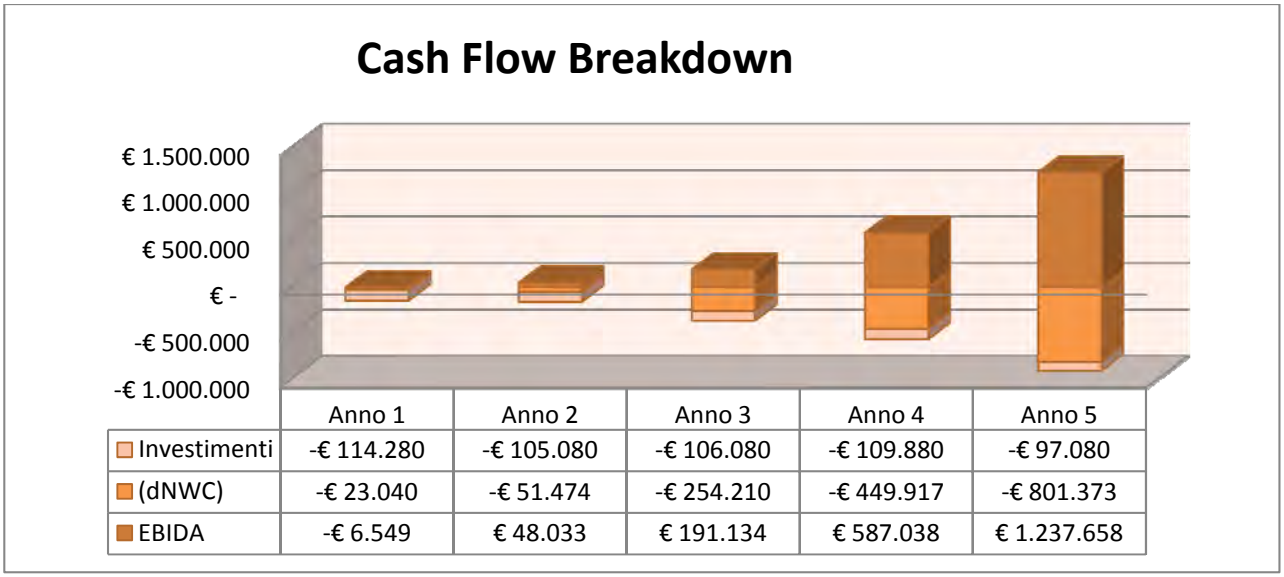
The analysis of expected cash flows in the five year period is described by the following chart:





The cash flow profile in the “best case” scenario shows the ability to generate cash from the fourth year onwards, reaching a value of approx. 340,000 € in the fifth year, corresponding to approx. 21% of EBITDA. On the other hand, in the "worst case" scenario, difficulties in penetrating the market substantially reduce liquidity in the latter years, postponing a positive cash flow to year five. The deterioration in the fourth year is caused by the fact that sales do not show expected growth compared to the third year and the company's expansion plan, which will nevertheless go ahead, has a negative impact.

Overall, the entrepreneurs expect a total cash outlay of approx. 420,000 € in three year in the “best case” scenario and just over 640,000 € spread over 4 years in the “worst case”. These values correspond to a monthly average burnout rate of 11,700-13,300 €.



The chart above shows that the investment plan defined by Bioexpansys is relatively constant over time since it is attributable to research activities. In detail, these expenses can be broken down into two distinct sections: investments in the first two years are aimed at product development and industrialization while expenditures in the following years are aimed at a future expansion of the product range. The latter do not generate revenue streams in the period under consideration but are necessary for the creation of value over time and to ensure a development pipeline and expansion of the product range for sustainability of the business over time.

The absorption of large amounts of cash attributable to changes in working capital is naturally caused by the imbalance between supplier and customer payment days, but it is necessary and desirable for the expansion of sales.

With regard to the appropriate financing plan for the startup, the entrepreneurs deem it necessary to raise funds in three different rounds. The first two rounds are necessary to achieve the final version of the product and its industrialization. The objective of the last stage is intended to cover the overhead costs necessary for market entry and the partial financing of subsequent research costs. It was decided to split the development requirements into two rounds in order to reduce the risk for investors and link their subsequent funding to the achievement of prototyping objectives. The potential investors in the startup are assumed to be, first of all, the entrepreneurs, Biomicron Srl and any interested business angels with expertise in the industry. In parallel, it is assumed that part of the requirement is covered by public funding allocated by competition and by participation in other tenders and competitions for startups (business plan competitions, etc.).

Best Case	Round A	Round B	Round C
Funding by competition	€ 30.000	€ 30.000	€ -
Biomicron Srl	€ 110.000	€ 80.000	€ 150.000
Business Angel	€ 20.000	€ 20.000	€ 40.000
Worst Case	**Round A**	**Round B**	**Round C**
Funding by competition	€ 30.000	€ 20.000	€ -
Biomicron Srl	€ 100.000	€ 70.000	€ 160.000
Business Angel	€ 20.000	€ 10.000	€ 40.000

As it can be seen from the above table, the first two funding rounds are identical since the planned process to industrialize the finished product does not change. The amount of the third round, on the other hand, depends on expected sales and, as a consequence, on self-financing flows; in the event of modest market success in the 3rd and 4th year, additional liquidity must be provided to support expansion and cost coverage in the fourth year. The values shown are based on the assumption of being able to finance only 20% through prizes and public funding and an amount not exceeding approx. 70,000 € from angel investors.

7. Conclusions and future perspectives

This work aims at assessing a development model for tissue engineering products.

The activities described in the previous paragraphs can be resumed in the following points:

- basic study on scaffolds for tissue engineering;
- basic study on bioreactors for tissue engineering;
- upgrade of a bioreactor for soft tissue engineering and fitting of this bioreactor to cadiomyocyte cultures on cardiac patch;
- design and development of a prototype of bioreactor for cell culture on hydrogel microspheres;
- submission of a National project for the development of resorbable functionalized membranes (class III) for oral implantology
- submission of an European project for the development of resorbable (class III) and non resorbable (class IIa) functionalized membranes for oral implantology;
- submission of a National project for the development of an innovative bioreactor for test of cadiomyocytes on cardiac patches (class I);
- submission of a Regional project for the development of an innovative bioreactor for engineered blood vessels (class IIa);
- submission of a Regional project for the development of an innovative bioreactor for adhesion tests of osteoblasts with advanced sensors (class I);
- elaboration of the business plan and foundation of a spin-off of Politecnico di Torino aimed at designing and developing bioreactors for tissue engineering.

The basic study on scaffolds for tissue engineering led to a deep understanding of microscopic scaffold features and to a computational characterization of different kind of scaffolds, both commercial and expressly built.

A bioreactor for soft tissue engineering previously having no control system has been activated and upgraded, building tailored electronic control and sensor conditioning circuits.

A low gravity bioreactor for culture of cells on hydrogel microsphere has been designed, within the Industrial Bioengeneering Group of the Department of Mechanics of Politecnico di Torino, and a prototype has been built.

Both bioreactors have been proved to work properly, even if no test with cells has been carried out yet. Both of them have been used in the European FP7 project BIOSCENT.

An in-depth analysis of tissue engineering market has been carried out, leading to the decision of developing scaffolds (namely, three kind of membranes for oral implantology) and bioreactors (namely two bioreactors for cardiovascular application and a bioreactor for orthopaedic application), resulting these two classes of products also affordable from a regulatory point of view.

Then, five research and development projects have been written and submitted, two for the development of membranes for oral implantology, namely B^3-BARRIER (National project) and BIO-PATH (European project), and three for the development of bioreactors for tissue engineering, namely PROBING (National project), ProVaDeRiVaSa (Regional project) and BIOBONE (Regional project).

Eleven entities, both private and public, have been involved in these projects. Table 34 summarizes them all.

Table 34: Summary of entities involved in projects.

	Kind of entity – Country	Projects
	SME - Italy	B^3-BARRIER, BIO-PATH, PROBING, ProVaDeRiVaSa, BIOBONE
	University - Italy	B^3-BARRIER, BIO-PATH, PROBING, BIOBONE
	University - Turkey	B^3-BARRIER
	Public Entity -Italy	B^3-BARRIER, BIOBONE
	SME - Spain	BIO-PATH
	Public Entity – Italy	BIO-PATH
	Private Research Centre - Spain	BIO-PATH
	University – Italy	PROBING, BIOBONE
	University – United Kingdom	PROBING
	SME – Italy	BIOBONE

	SME – Italy	ProVaDeRiVaSa

Three of the projects submitted (B^3-BARRIER, BIO-PATH and PROBING) have been already approved and financed, while two of them (ProVaDeRiVaSa and BIOBONE) are under evaluation.

The three project approved, involving 146 person months, have a total value of €1.065.190,00, financed by public entities with a €493.076,00 contribution. Table 35 summarizes their statistics.

Table 35: Summary of statistics of the three projects approved.

Project acronym	Person months involved	Project value	Public contribution granted
B^3-BARRIER	32	€ 240.000,00	€ 120.000,00
BIO-PATH	78	€ 575.190,00	€ 248.076,00
PROBING	36	€ 250.000,00	€ 125.000,00
Total	146	€ 1.065.190,00	€ 493.076,00

The two projects that are still under evaluation, involving 84 person months, have a total value of €681.600,00; the contribution requested for them is €472.960,00. Table 36 summarizes their statistics.

Table 36: Summary of statistics of the two projects now under evaluation.

Project acronym	Person months involved	Project value	Public contribution requested
ProVaDeRiVaSa	21	€160.000,00	€120.000,00
BIOBONE	63	€ 521.600,00	€ 312.960,00
Total	84	€ 681.600,00	€ 432.960,00

Defining an index “rate of financial success” as the ratio between the funding granted and the requested one, the rate of financial success of this operation is 100% till now.

This unquestionably shows that the model of fund raising developed perfectly works.

Moreover, an industrial start-up, spin-off of Politecnico di Torino, named Bioexpansys s.r.l., was founded and its business plan was successfully elaborated, in cooperation with the incubator I3P inside Politecnico di Torino.

This business plan shows that, in the best case, against potential revenues of more than €3.000.000,00 at its fifth year of activity, and an EBITDA up to 44%, a fund raising of about €420.000,00 is needed by its third year. By now, €35.000,00 have been produced, by submitting Bioexpansys’ business plan for Regione Piemonte’s (€15.000,00) and Istituto Superiore Mario Boella’s (€20.000,00) contribution.

As future perspectives, a few key points can be underlined:

- the prototype of bioreactor for cell culture on hydrogel microsphere must be industrialized and transformed in a product marketable by Bioexpansys, being careful at properly exploiting the patent filed, in case of its approval;

- the projects submitted and approved must be correctly driven in order to demonstrate the effectiveness of outsourcing R&D activities for cutting-edge products, as tissue engineering products are;
- certification must be achieved for all the products under development;
- the products developed must be marketed by 2 (for scaffolds) to 5 (for bioreactors) years from now, being the time-to-market essential for a successful competition in this field;
- the increase in revenues by the SME involved must be significant and a conspicuous number of patents must be filed, being this aspect essential in high technology products;
- the spin-off/start-up Bioexpansys must be taken to breakeven point by its third year of activity and to its full revenues by its fifth year of activity.

In conclusion, the business model proposed, even if only partially verified, is worth being pursued in the development of tissue engineering products.

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